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Centre européen d'étude du Diabète

# THESE

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## Amélioration de la viabilité des îlots pancréatiques dans le pancréas bioartificiel

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# ABBREVIATIONS

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**ADE** – adenosine  
**ADP** – adenosine diphosphate  
**ATP** – adenosine triphosphate  
**BSA** – bovine serum albumin  
**CREB** – c-AMP Response Element-binding  
**COX** – cyclooxygenase  
**DNA** – deoxyribonucleic Acid  
**ECM** – extracellular matrix  
**ELISA** – enzyme-linked immunosorbent assay  
**ESCs** – embryonic stem cells  
**FDA** – fluorescein diacetate  
**GLUT** – glucose transporter  
**GSIS** – glucose stimulated insulin secretion  
**HBSS** – Hank's buffered saline solution  
**HIF-1 $\alpha$**  – hypoxic inducible factor  
**HRP** – horseradish peroxidase  
**IAK** – islet after kidney  
**IBMIR** – instant blood mediated inflammatory reaction  
**IEQ** – islet equivalents  
**IL** – interleukin  
**ITA** – islet transplantation alone  
**KRB** – krebs ringer solution  
**MCP-1** – monocyte chemoattractant protein-1  
**mRNA** – messenger ribonucleic acid  
**NSAIDs** – non-steroidal anti-inflammatory drugs  
**NF- $\kappa$ B** – nuclear factor-kappa B  
**NO** – nitric oxide  
**PAK** – pancreas after kidney  
**PBS** – phosphate buffered saline  
**PCR** – quantitative polymerase chain reaction  
**PFC** – perfluorocarbons  
**PGE<sub>2</sub>** – prostaglandins  
**PI** – propidium iodide  
**PO** – oxygen pressure  
**PTA** – pancreas transplantation alone  
**PVDF** – polyvinylidene difluoride  
**ROS** – reactive oxygen species  
**SPK** – simultaneous pancreas kidney  
**STZ** – streptozotocine  
**TBS** – tris buffered saline  
**TLM** – two layer method  
**UWS** – university wisconsin solution  
**VEGF** – vascular endothelial growth factor

# 1 INTRODUCTION

## 1.1 Pancreas

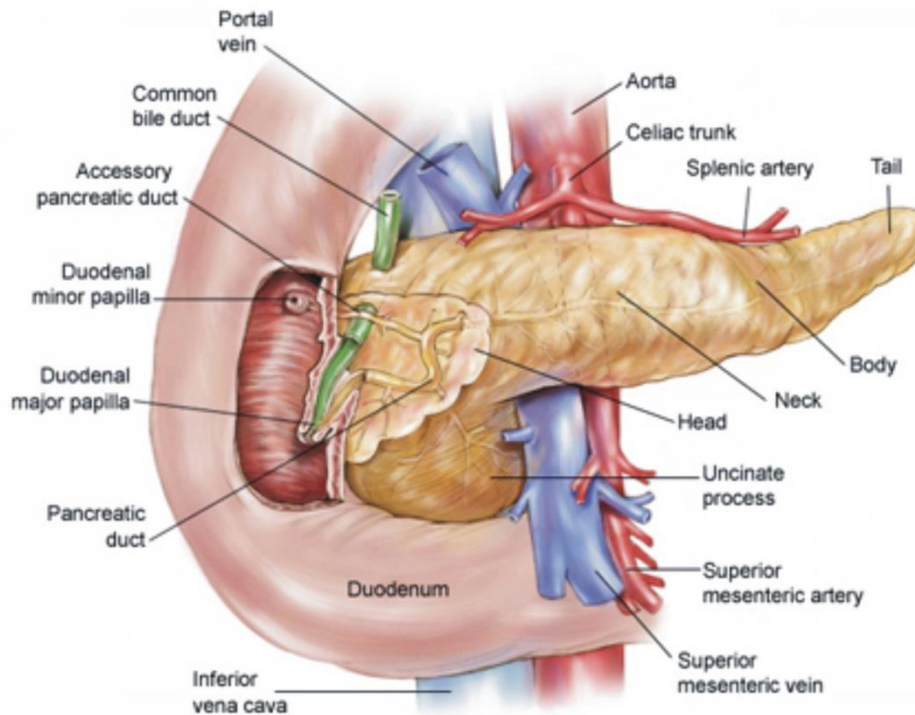
### 1.1.1 Anatomy

The pancreas is a retroperitoneal digestive organ connected to the duodenum through the pancreatic duct. It has 15cm length and around 70-80g of weight. It is located in the front of the aorta and behind the stomach.

Anatomically, the pancreas is divided in 4 parts: head, neck, body and tail (**Fig. 1-1**). The head is situated in the concavity of the duodenum, the body is placed just behind the stomach, and the tail is next to the spleen. The duodenum and the pancreas are irrigated from the celiac trunk and the superior mesenteric artery. The body and tail of the pancreas are supplied mostly by branches of the splenic artery, namely the great, superior, caudal, and dorsal pancreatic arteries.

The pancreas is a heterocrine gland with an exocrine and an endocrine tissue. The exocrine tissue represent between 98% and 99% of the pancreas by weight while endocrine tissue makes up the other 1 to 2%. The endocrine tissue is made of islets (pancreatic or Langerhans) which will be detailed thoroughly later in the manuscript. The exocrine tissue is arranged in acini, acinar cell clusters surrounding tiny ducts. Acinar cells produce and release digestive enzymes into the ducts. The ducts of many acini connect to form larger ducts which run into the main pancreatic ducts [1].

The pancreas has two main ducts: the major pancreatic duct (duct of Wirsung or pancreatic duct) and the accessory pancreatic duct. The function of these ducts is the delivery of pancreatic secretions to aid digestion into the duodenum. The main pancreatic joins the common bile duct right before entering the duodenum, so they both dump their contents via the greater (major) duodenal papilla (comprised of the ampulla of Vater, or hepatopancreatic ampulla, and the sphincter of Oddi). The main pancreatic duct crosses the whole pancreas collecting the pancreatic juice from all acinar cells. The accessory duct drains into the duodenum via the lesser (minor) duodenal papilla [1, 2].



**Figure 1-1 Pancreas anatomy**  
 Source: <http://www.aboutcancer.com>

## 1.1.2 Function

The pancreas because of the mixed tissue has two complementary functions. The exocrine tissue is involved in digestion and the endocrine tissue involved in glycemia regulation [2].

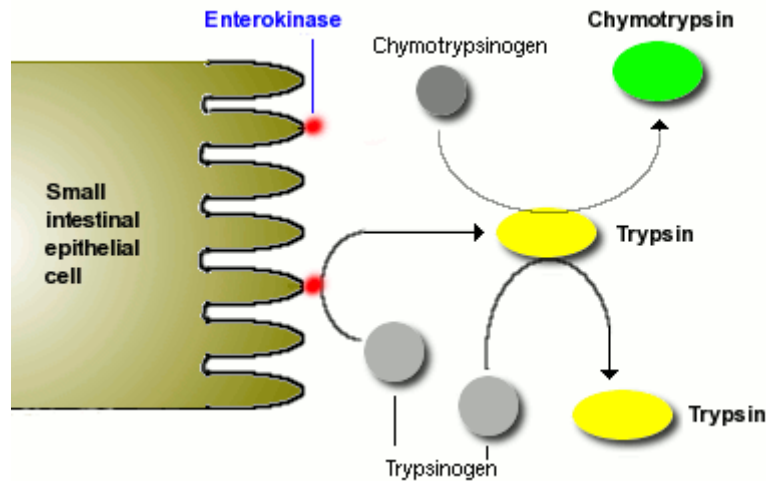
### 1.1.2.1 Exocrine tissue

Exocrine tissue is composed of acinar cells, ducts and centroacinar cells. The ducts and the centroacinar cells produce bicarbonate and water to insure the flush of the pancreatic juice in the ducts [3]. All enzymes secreted by the pancreas have the capacity to reduce digestible macromolecules into small molecules able of being absorbed [4].

For an efficient digestion three groups of enzymes are needed:

Proteases: Different proteases, like pepsin, are synthesized in the pancreas and secreted into the lumen of the small intestine, and then the digestion of protein is initiated in the stomach by the pepsin. Trypsin and chymotrypsin are the most important pancreatic proteases. These enzymes are synthesized as an inactive proenzymes trypsinogen and chymotrypsinogen which are packaged into secretory vesicles. The trypsinogen is activated by the enterokinase and is converted in the active form, trypsin. Immediately chymotrypsinogen is activated in chymotrypsin by trypsin. This activation occurs in the lumen of the small intestinal in order to do the protein digestion (**Fig. 1-2**). When the pancreatic secretions reach the small intestine, all the proteases are active and the digestion process starts [4]. The function of trypsin and

chymotrypsin is the digestion of proteins into peptides and peptides. There are other proteases also from the pancreas like carboxypeptidases which are able to digest the peptides and proteins into single amino acids. However, the digestion into single amino is mainly due by peptidases which are on the surface of small intestinal epithelial cells [4].



**Figure 1-2 Activation of Trypsin and Chymotrypsin [4]**

Pancreatic lipase: triglycerides are the major component of the diet and the intestinal mucosa is not able to absorb directly this molecule. Triglycerides are hydrolyzed by the pancreatic lipase which converts the triglycerides in monoglycerides and free fatty acids. Both lipases are needed to get a proper digestion of triglycerides producing free fatty acids and monoglyceride that can be absorbed.

Amylase: this enzyme is present in the pancreatic secretions and in the saliva. Its function is the starch hydrolysis into maltose, trisaccharide maltotriose and small fragments called limit dextrans [4].

#### 1.1.2.2 Endocrine tissue

The endocrine portion of the pancreas takes the form of small clusters of cells called islets of Langerhans or pancreatic islets. Islets produce and release important hormones directly into the bloodstream. Two of the main pancreatic hormones are insulin, which acts to lower blood sugar, and glucagon, which acts to raise blood sugar. Maintaining proper blood sugar levels is crucial to the functioning of key organs including the brain, liver, and kidneys. Human pancreas contains around one million of islets [5].

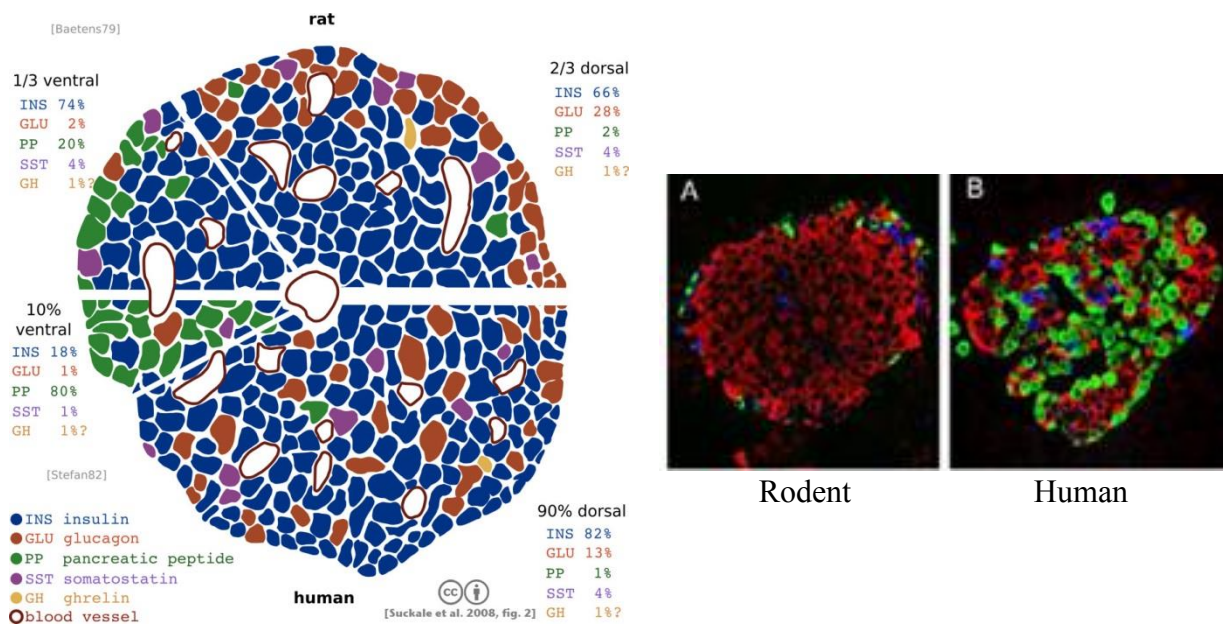
Pancreatic islets are composed of five cell types; each type produces a different endocrine product and has a specific role [5]:

- $\alpha$  cells are responsible for the glucagon secretion (hyperglycemic hormone) and represent around 15-20% of the total tissue. These cells have dense secretory vesicles. The release is regulated by the influence of different factors (nutritional factors, hormones and neurotransmitters).
- $\beta$  cells produce insulin (hypoglycemic hormone) and are the most abundant of the islet, around 60 to 80% of endocrine cells. Insulin is stocked in hexamers inside vesicles recognized by the crystalline core surrounded by a nimbus. Insulin secretion

is regulated by the synergic actions of nutritional factors, hormonal messengers and nervous.

- $\delta$  cells secrete somatostatin and represent around 5% of the endocrine cell population. Somatostatin cells are located in many species in the  $\beta$  cells periphery because there is a close link between them. The role of the somatostatin is to inhibit the pancreas secretion with the inhibition of certain enzymes like insulin and glucagon.
- PP cells contain pancreatic polypeptides, are located in the periphery related to  $\beta$  cells and represent the 1% of the endocrine cells. Pancreatic polypeptides play an important role in the regulation of insulin secretion, increasing basal insulin concentration in plasma but do not affect glucose stimulated secretion of insulin or glucagon.
- Ghrelin cells produce the peptide hormone ghrelin and represent 1% of endocrine cell in adult pancreas. Ghrelin play a role in glucose-stimulated insulin secretion [6].

Depending upon the species, the distribution of the different cell types is different. In rodents, islets have a well-defined structure with a central core of  $\beta$  cells (representing 60–80% of islet cells) and a layer of other endocrine cells surrounding the core including  $\alpha$  cells (15–20%),  $\delta$  cells (<10%) and PP cells (<1%). In human islets, the  $\alpha$ -,  $\beta$ - and  $\delta$ - cells appear to be randomly distributed throughout the islet. The proportion of  $\beta$  cells is higher in rodent islets than in human cells, 77% vs. 55% [7] (**Fig. 1-3**).



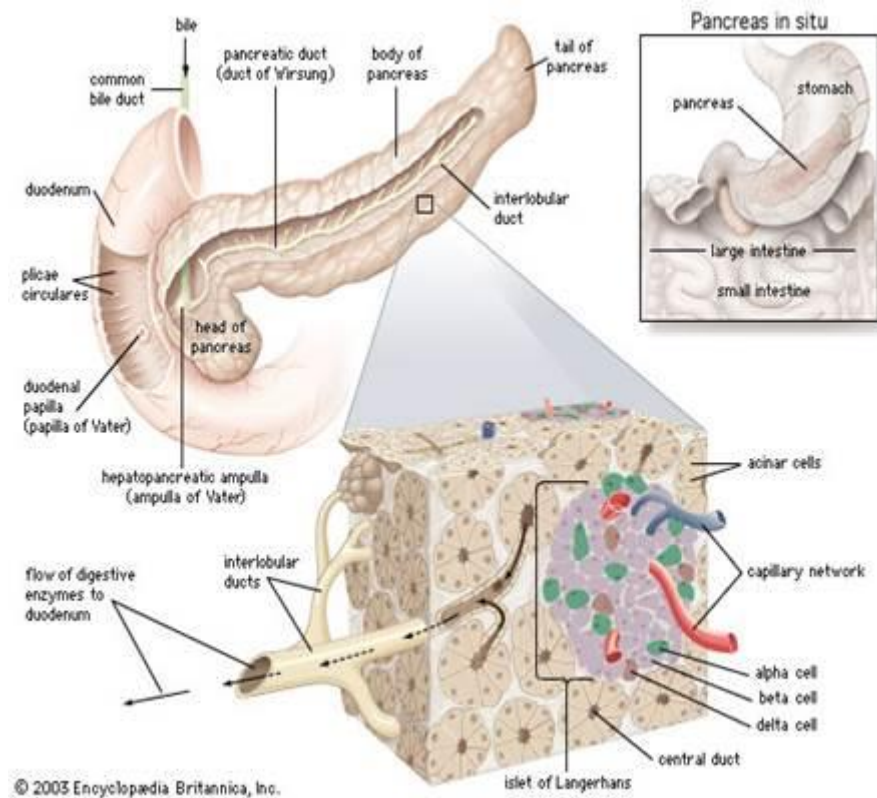
**Figure 1-3 Human and rodent pancreatic islets of Langerhans**  
 Source: Suckale et al. 2008 (left), Diabetes Research Institute, Miami (right)

### 1.1.2.3 Islet vascularization

In the pancreas, islets are richly vascularized, which permits a fine tuning in glucose detection in blood and the subsequent insulin release. Although islets represent only 1-2% of the mass of the pancreas, they receive about 10 to 15% of the pancreatic blood flow. Capillaries surrounding islets show a remarkable number of small pores called fenestrates. Via these structures, nutrient exchanges occur, glucose permeate through the capillaries is detected by  $\beta$  cells, which exert glycemic control in response. Additionally, they are



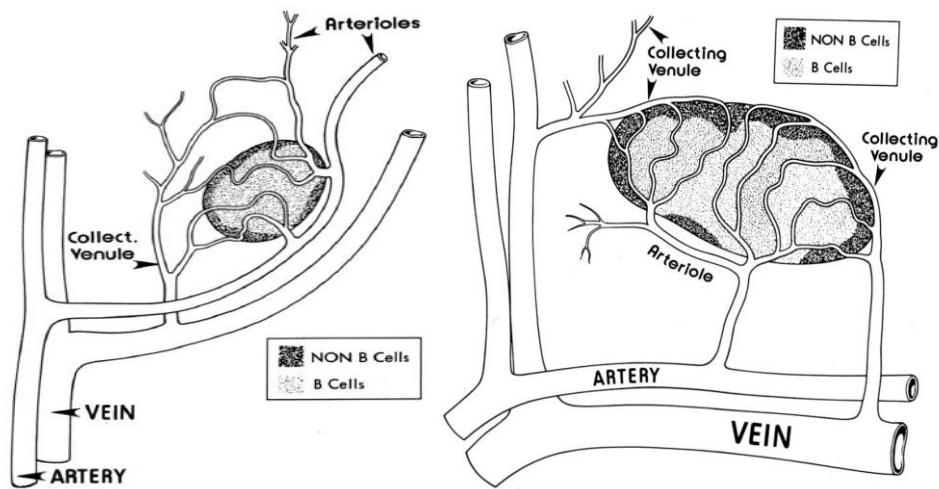
innervated by parasympathetic and sympathetic neurons, and nervous signals clearly modulate secretion of insulin and glucagon [5] (**Fig. 1-4**).



**Figure 1-4 Islet vascularization in the pancreas**

Source: Encyclopedia Britannica, 2003

The organization of islet vascularization depends on islet size. Afferent arterioles are connected with acinar and ductular microvessels forming insulin-acinar portal system. Small islets (60-160  $\mu\text{m}$  diameter) are not closely associated with duct or vessels. Intermediate islets (160-260  $\mu\text{m}$  diameter) are found along secondary vessels and large islets (260-800  $\mu\text{m}$  diameter) are often clumped at a major branches of blood vessels and in close association with major ducts. Small islets have one afferent vessel, an arteriole and intermediate of large big ones have got 1 to 3 short arterioles entering the islet directly from the large artery/arteriole. From the interior of this structure emerge efferent capillaries that extend beyond the islet periphery (**Fig. 1-5**) [8].



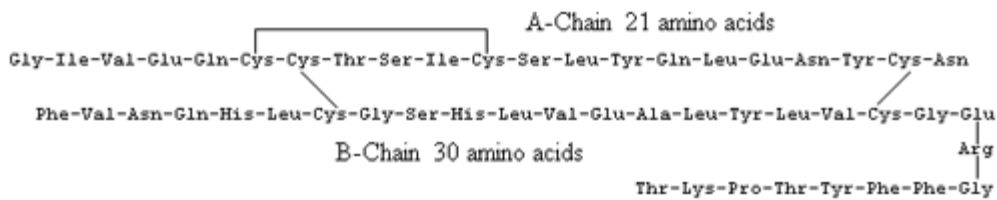
**Figure 1-5 Microvascularization of small and large islet [8]**

Through the endocrine capillaries, islets are relatively over-perfused under basal conditions, and their blood supply regulation is independent of the exocrine pancreas. Pancreatic islets are highly metabolically active, depending on oxygen consumption and glucose oxidation for their formation of ATP and subsequent insulin secretion. The highly specialized glomerular-like vascular structure of islets greatly facilitates proper glucose sensing of ambient blood glucose concentration and the distribution of secreted hormones to target organs. The mechanisms that have evolved to regulate islet perfusion are complex and mediated by signals from the nervous, hormonal, and circulatory systems. Nutrients are known regulators of islet function. Glucose is the most important insulin secretagogue, and glucose administration almost doubles islet perfusion through multiple mechanisms. A suggested mechanism for this phenomenon is that immediate or anticipatory islet vasodilation is mediated by the nervous system, followed by maintenance and more precise regulation of islet perfusion by locally produced factors. Similarly, induction of hypoglycemia by insulin administration leads to an immediate increase in islet blood flow, presumably to promote glucagon influx into the circulation [9]. The capillary density combined with a high blood perfusion of the islets delivers high amounts of oxygen to the islets, keeping  $PO_2$  metabolically active islet tissue in equilibrium to that of venous blood (40 mmHg), and permit the secretion of insulin [10-12].

## 1.2 Insulin

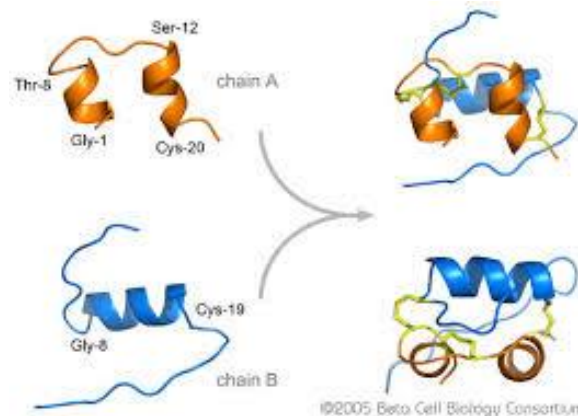
### 1.2.1 Structure

Circulating and biologically active insulin is monomeric and its molecular weight is 5800 Da. It is composed of two polypeptide chains: chain A has 21 amino acids and chain B has 30 amino acids (in humans). Two disulfide bridges covalently link the chains and chain A is characterized by an internal disulfide bridge (**Fig. 1-6**). The positions of these three disulfide bonds are invariant in mammalian forms of insulin. At micromolar concentrations, insulin dimerizes, and in the presence of zinc, it further associates into hexamers [8].



**Figure 1-6 Basic structural parameters of mature human insulin**  
 Source: <http://dailymed.nlm.nih.gov>

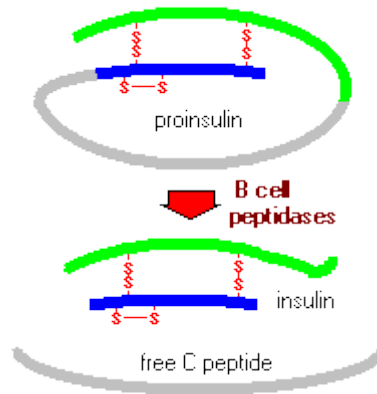
In the core of the protein there is a cluster of hydrophobic residues which helps the protein stability using the constraint of the polypeptide backbone by the disulfide bridges. Outside the core there is a first flat and aromatic nonpolar surface buried upon dimer formation contributing to an antiparallel sheet structure. The second nonpolar surface is more extensive and is buried upon hexamer formation (**Fig. 1-7**). The surface that insulin uses for binding to his receptor is the same surface used for self-assembled [8].



**Figure 1-7 Quaternary arrangement of insulin**  
 Source: <http://www.cell.org>

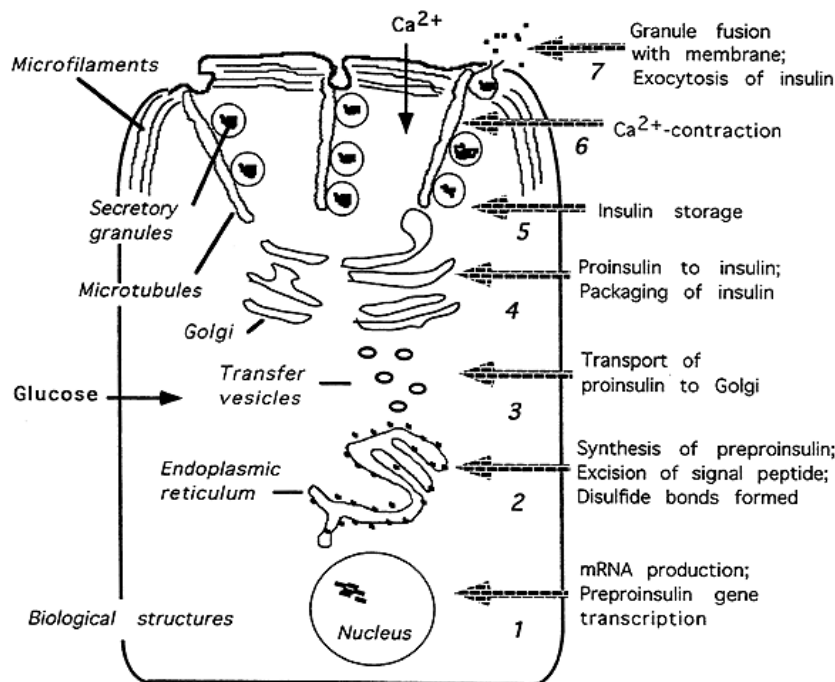
### 1.2.2 Insulin biosynthesis

Insulin is a hormone produced exclusively by  $\beta$  cells in the pancreas. The insulin mRNA is translated as an inactive protein precursor called preproinsulin which contains N-terminal signal peptide. The N-terminal signal peptide interacts with the signal recognition particle (SRP), which facilitates the entry of the preproinsulin in the rough endoplasmic reticulum (RER) from the cytosolic compartment. The N-terminal signal sequence is cleaved to generate proinsulin. It is composed of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle known as the C peptide. In the lumen of the ER, proinsulin undergoes protein folding. The folded proinsulin and C peptide are then delivered to the Golgi apparatus and packaged in secretory granules [8] (**Fig. 1-8**).



**Figure 1-8 Mechanisms of conversion of proinsulin to insulin**  
 Source: Bowen, 1999

The conversion of proinsulin into insulin takes place in the secretory granules, where insulin is stored. Insulin is secreted from the  $\beta$  cells in the pancreas by exocytosis and circulates into islet capillary blood. The C peptide is also secreted into blood, but has no known biological activity [8] (**Fig. 1-9**).



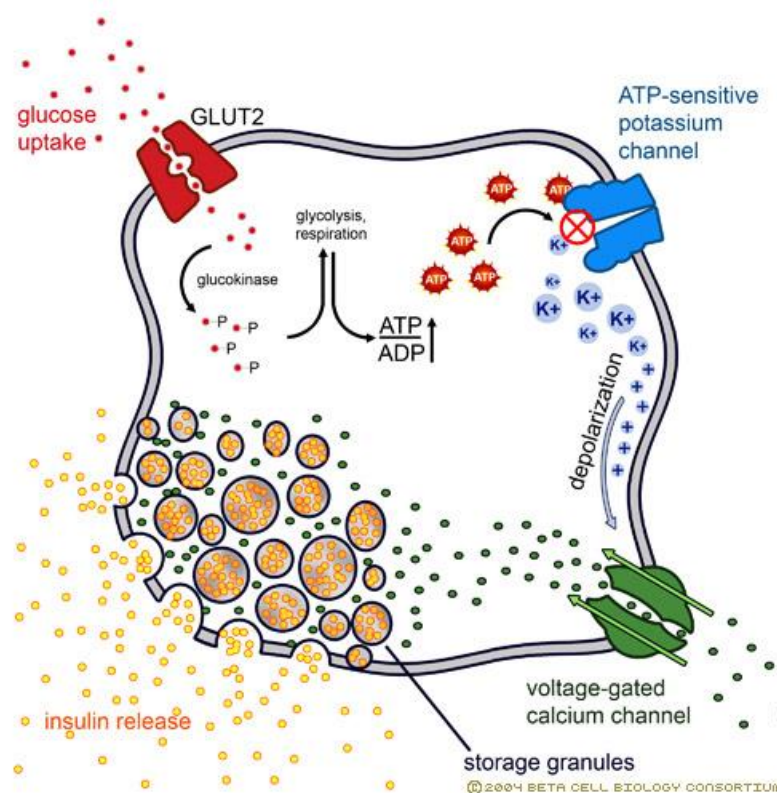
**Figure 1-9 Intracellular steps of insulin secretion**  
 Source: [http:// www.nbs.csudh.edu](http://www.nbs.csudh.edu)

### 1.2.3 Control of insulin secretion

Glucose stimulation is detected thanks to sensing devices able to measure circulating glucose like the glucose transporter 2 (GLUT2), constitutively expressed in  $\beta$  cells. Glucose is internalized in  $\beta$  cells after mobilization of GLUT2 to the plasma membrane; this mechanism is insulin-independent ensuring high glucose influx. After entering, glucose is phosphorylated

by the glucokinase, which acts as a glucose sensor because of its ‘low’ affinity for glucose and becomes active only when glucose concentration increases. Glucokinase catalyzes the phosphorylation of the glucose in glucose-6-phosphate. This product is then transformed into pyruvate, which is then oxidized through the tricarboxylic acid cycle by mitochondria in  $\beta$  cells to produce ATP [13].

Production of ATP and increase in ATP/ADP ratio results in closure of ATP dependant potassium ( $K_{ATP}$ ) channels. Accumulation of potassium ions increased the positive charges in the vicinity of the membrane which triggers its depolarization. The change in membrane polarization leads to the activation in the voltage-gated calcium channels and the entry of calcium ions into the cell. The increase of the influx calcium concentrations produced the fusion of insulin granules with the plasma membrane and exocytosis of granule content. Once insulin is exported from the  $\beta$  cells, it is diffused in the blood vessels to control the blood glucose levels providing the first pic in insulin secretion [8] (**Fig. 1-10**).



**Figure 1-10 Insulin secretion**  
 Source: <http://www.ciitn.missouri.edu>

Then, the level of insulin go back to basal levels and after 10 minutes of glucose stimulation, the second phase of secretion starts (during 45 minutes). The second phase slower than the first phase explains the biphasic response in the insulin secretion curve (**Fig. 1-11**) [14].

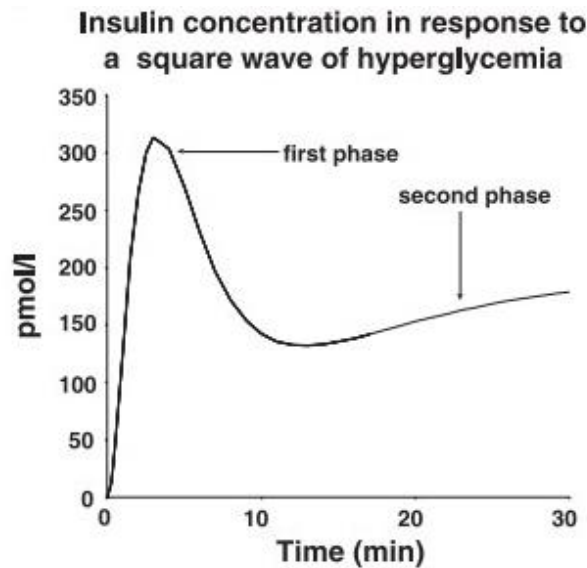
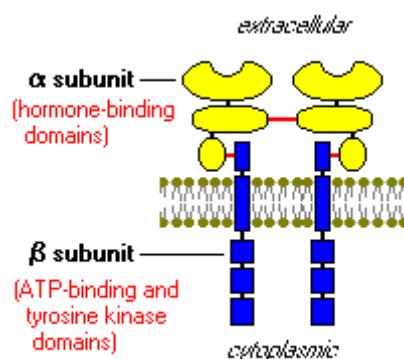


Figure 1-11 Insulin secretion curve [14]

#### 1.2.4 Insulin receptor: structure and action

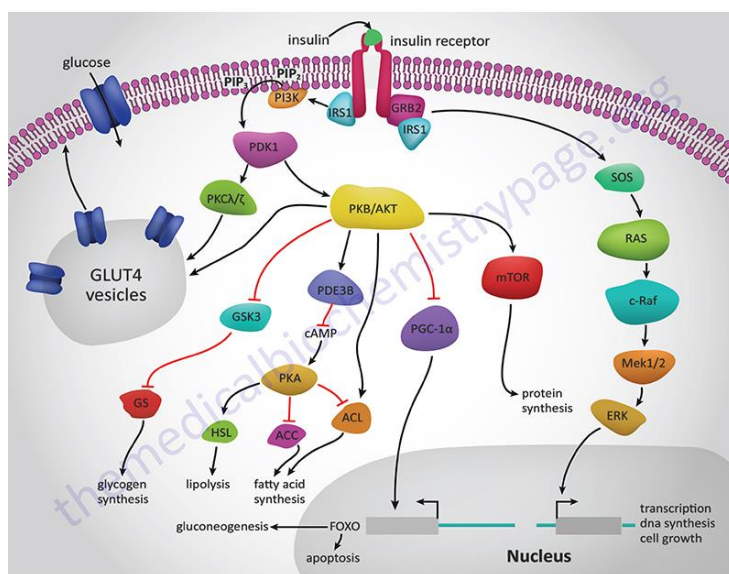
Once release into the blood stream, insulin circulates in blood until meet its receptor. The main organ that predominantly clears insulin from circulation is the liver. In a non-diabetic patient the liver clears about 60% of endogenous insulin via the hepatic portal vein. Once glucose is transported inside the hepatocytes, insulin stimulates glycogen synthesis and storage and inhibits glucose production (glycogenolysis) [8].

The insulin receptor is a tyrosine kinase, an enzyme family whose members play critical regulatory roles in development, cell division, and metabolism. The insulin receptor is composed of two  $\alpha$ -subunits and two  $\beta$ -subunits linked by disulfide bonds located in the plasma membrane. The  $\alpha$ -subunit has a molecular mass of 130 kDa and contains the insulin binding domains located in both sites of a rich cysteine sequence which allows the formation of disulfide bonds. In the absence of insulin, the  $\alpha$ -chains have an inhibitory function and the receptor is in an inactive configuration. The  $\beta$ -subunit has a molecular mass of 95 kDa and is composed by three compartmentalized regions: the extracellular, transmembrane and cytosolic domains. The cytosolic domain has ATP-binding and tyrosine kinase activity with a regular loop which covers the catalytic site and keeps the receptor inactive (**Fig. 1-12**). When insulin binds to the  $\alpha$ -subunit, there is induction of the tyrosine autophosphorylation of the receptor  $\beta$ -subunit, which in turn is activated and phosphorylates its substrates, among which IRS-1 [8].



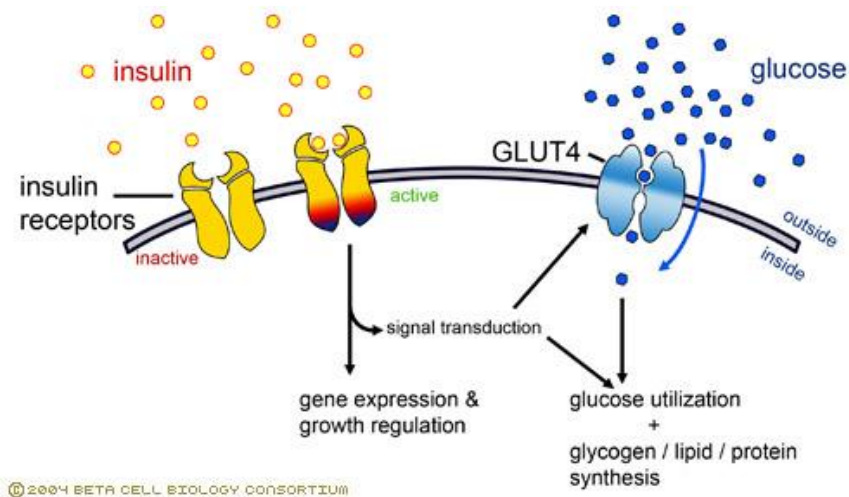
**Figure 1-12 Insulin receptor:  $\beta$  and  $\alpha$  subunits**  
 Source: <http://www.vivocolastate.edu>

IRS-1 acts as a type of docking center for recruitment and activation of other enzymes implicated in insulin's pleiotropic action. For instance, IRS-1 interacts with and recruits phosphatidylinositol (PI) 3-kinase. These events lead in particular to activation signaling pathways required for insulin as GLUT4 membrane translocation [15] (**Fig. 1-13**).



**Figure 1-13 Different cellular effects of insulin**  
 Source: <http://themedicalbiochemistrypage.org/insulin.php>

Insulin-stimulated glucose uptake is achieved by insulin-sensitive glucose transporters (GLUT4) present on the plasma membrane of muscle cells, adipocyte, hepatocytes and other targeted tissues. When glucose and circulating insulin decrease, the GLUT4 transporters shift back to the storage vesicles waiting for future insulin signaling (**Fig. 1-14**). In skeletal muscles, glucose uptake depends on the coordination of three steps: (1) increased delivery of glucose to the muscle fiber by increased blood flow, (2) increased glucose transport across the plasma membrane (GLUT4 and other facilitative transporters), and (3) phosphorylation of glucose by hexokinase [16].



**Figure 1-14 Insulin-mediated glucose uptake**  
 Source: cell biology consortium

Insulin signaling ends with the degradation of insulin and the dephosphorylation of the receptor. Insulin-receptor complexes are internalized together with other receptors through the formation of clathrin-coated vesicles, and then complexes are delivered to endosomes, where the acidic pH induces the dissociation of insulin molecules from insulin receptors. Subsequently, insulin molecules are targeted to late endosomes and lysosomes where they are degraded and receptors are recycled back to the cell surface in order to be reused [17].

Once the glucose is entered in the cells, it is transformed and stored into glycogen. Glycogen synthesis (**Fig. 1-15**) is stimulated by insulin which inhibits and activates specific enzymes involved in this pathway. It is a very large branched polymer of glucose residues that can be broken down to yield glucose molecules when energy is needed. It consists in a glucose branch linked by  $\alpha$ -1,4-glycosidic bonds and is ramified in  $\alpha$ -1,6-glycosidic bonds every 7 from 11 residues. It is a polymer of chemical formula  $(C_6H_{10}H_5)_n$ . Glucose stock is produced by the glycogen synthetase. This enzyme avoids, after the digestion, the accumulation of glucose in the blood (hyperglycemia). When glucose is needed, degradation of glycogen in glucose (glycogenolysis) by the glycogen phosphorylase is triggered by the glucagon or adrenaline [8].



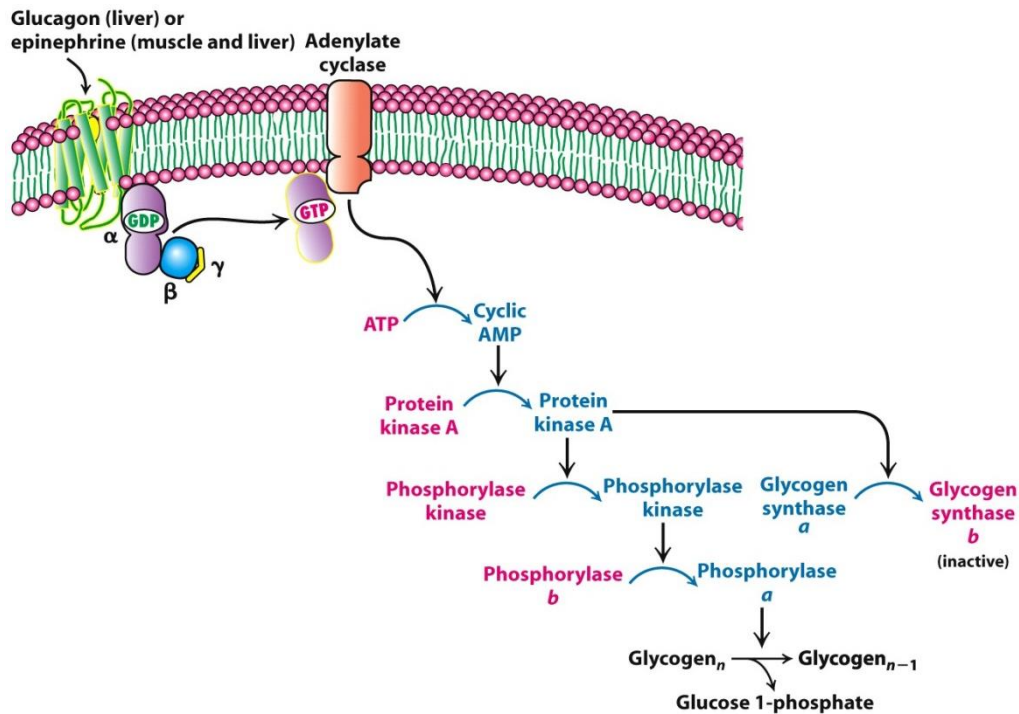


Figure 21.19  
 Biochemistry, Seventh Edition  
 © 2012 W. H. Freeman and Company

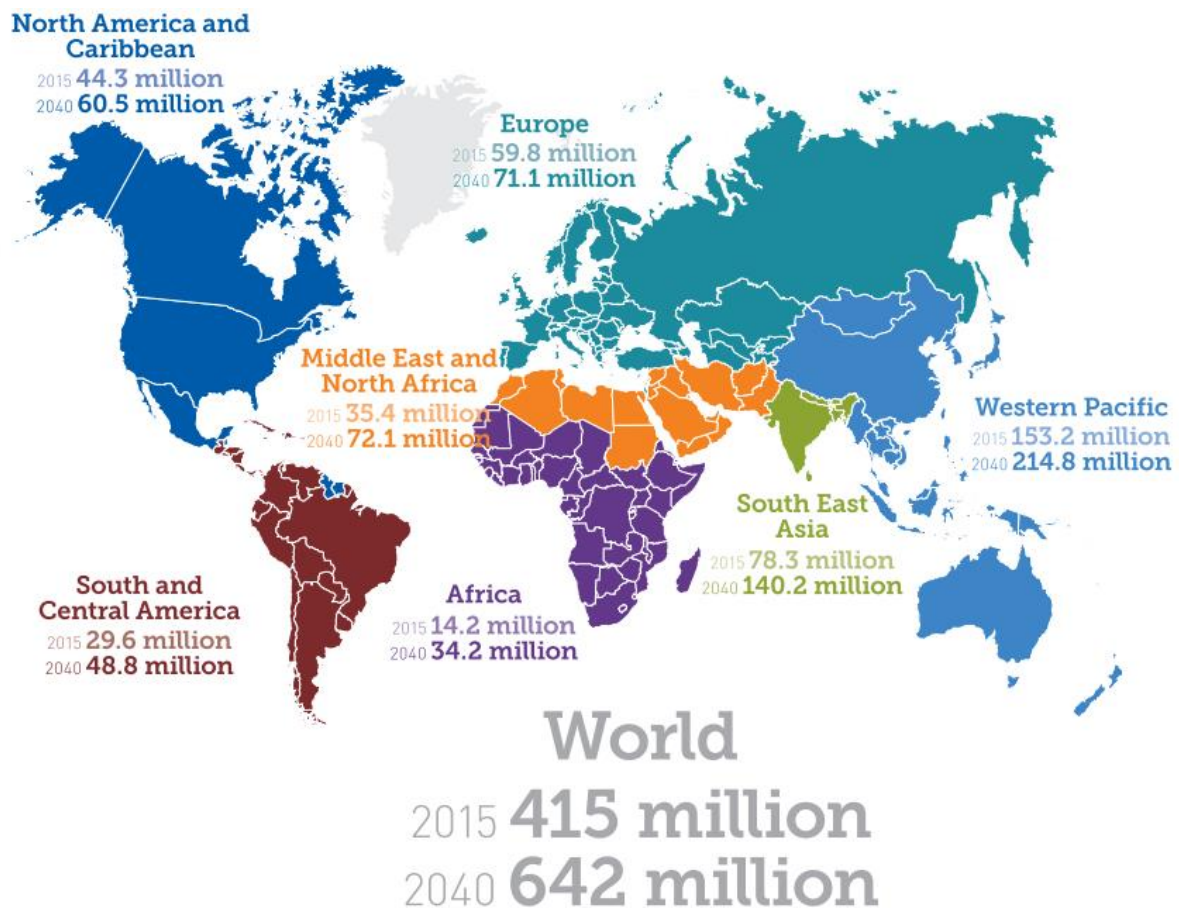
Figure 1-15 Mechanisms of glycogenesis  
 Source: Biochemistry, 2012

## 1.3 Diabetes

### 1.3.1 Definition criteria and classification of DM

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the  $\beta$  cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action [18]. The current World Health Organization (WHO) diagnostic diabetes when the fasting plasma glucose is higher than 7 mM (1.26g/L) or 11 mM (2g/L) two hours after hyperglycemia produced by oral route (OGTT).

According to the International Diabetes Federation (IDF), diabetes prevalence in 2015 worldwide was estimated than half a million children aged 14 and under living with type 1 diabetes. It is also estimated that actually there are 415 million adults aged 20-79 with diabetes worldwide, including 193 million who are undiagnosed. A further 318 million adults are estimated to have impaired glucose tolerance, which puts them at high risk of developing the disease. If this rise is not halted, by 2040 there will be 642 million people living with the disease (**Fig. 1-16**). There are different types of diabetes such as type 1, type 2, monogenic, secondary and gestational between others.



**Figure 1-16 Worldwide diabetes distribution prevalence in 2015 (IDF)**

### 1.3.2 The natural history of DM

Glucose is the main nutrient of the body's cells. Therefore every individual consumes continuous glucose (2 mg/min/kg on average); mainly muscle during inter period absorptions. The digestive tract becomes the main consumer in postprandial periods, reducing the glucose levels used by the brain. If exogenous glucose from the meals is not enough, there are stocks of glucose in the liver (75%) and in the kidney (25%). Normoglycemia is maintained when the glucose intake perfect fit for consumption, however, if it is not the case the insulin-glucagon system will be implicated.

This system does not work properly in some cases due to insulin deficiency and insulin resistance. As a consequence there is a reduction in glucose consumption and an increase in hepatic glucose production (as a result of insulin deficiency and excessive glucagon) and renal (by increasing tubular reabsorption of glucose).

The imbalance between the reduced consumption and increased production of glucose, results in hyperglycemia. Early symptoms of hyperglycemia such as frequent urination, thirst, blurred vision, fatigue and headache can develop a pre-diabetic status.

### 1.3.3 Diabetes diagnostic

The effects of high glucose concentration in blood are multiple and can be used for the diagnosis (**Table. 1-1**):

Test <sup>a</sup>	Threshold	Qualifier
Hemoglobin A <sub>1c</sub> or	≥ 6.5%	Lab NGSP-certified, standardized DCCT assay
Fasting glucose or	≥ 126 mg/dL (7.0 mmol/L)	No caloric intake for at least 8 hours
2-hour glucose or	≥ 200 mg/dL (11.1 mmol/L)	After 75 g of anhydrous glucose
Random glucose	≥ 200 mg/dL (11.1 mmol/L)	Plus classic hyperglycemia symptoms or crisis

NGSP, National Glycohemoglobin Standardization Program; DCCT, Diabetes Control and Complications Trial.

<sup>a</sup> Results must be confirmed by repeated testing.

**Table 1-1 Diagnostic criteria for diabetes**  
**Source: American Diabetes Association**

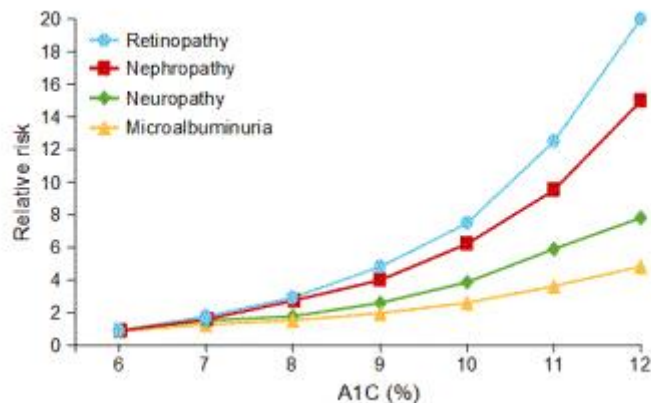
Fasting plasma glucose (FPG) test is the easiest and the first to be performed after the patient has gone at least 8 hours without food or drink. It is performed analyzing the blood glucose levels through a blood test.

The random plasma glucose (RPG) test is a simple blood sugar test. There is no need to fast for the RPG. The test is simple and can be performed taking a blood test and having the levels of glucose. A normal blood glucose level reading, without fasting first, of under 140 mg/dl is considered normal. However, a level of over 200 mg/dl, especially with symptoms of frequent urination, excessive thirst, etc. will indicate a strong possibility of diabetes [19].

What makes the FPG different from the RPG test is that through fasting the body of a person without diabetes will produce and process insulin in response to increased glucose in the blood. For a person with diabetes, the body will not have that same response and their blood glucose levels will remain high. Receiving two readings of blood glucose levels of 126 mg/dl or over usually means the person has diabetes. A normal level for a person without diabetes should be in the 70-110 mg/dl range after fasting [19].

The A1C test is based on the percentage of the hemoglobin, the protein that carries oxygen, is coated with glucose (glycated). In the body, red blood cells are constantly forming and dying, but typically they live for about 3 months. Thus, the A1C test reflects the average of a person's blood glucose levels over the past 3 months. The A1C test result is reported as a percentage. The higher the percentage, the higher a person's blood glucose levels have been. A normal A1C level is below 5.7 %. The test plays a critical role in the management of the patient with diabetes, since it correlates well with both microvascular and, to a lesser extent,

macrovascular complications and is widely used as the standard biomarker for the adequacy of glycemic management (Fig. 1-17).



**Figure 1-17 Microvascular complications associated with A1C increase**  
**Source: Internal Medicine & Pediatric Associates**

The last test given is called an oral glucose tolerance test. The patient needs to fast, usually overnight, for 10-16 hours before the test is taken. First blood sample is performed to give a basal reading. Then, the patient is given a cola drink with high content of sugar (75 grams of glucose) and blood test is taken 2 hours after the drink. The two blood tests after the drink with high glucose content will show the rise and fall of blood sugar levels over time, as a normal profile [19]. In the first measure, a normal level should be in the 70-110 mg/dl range for non-diabetic and >150 mg/dl for diabetic person. The second measure after 2 hours, a normal level should be >140 mg/dl range for non-diabetic and >200 mg/dl for diabetic person.

There are two principal types of diabetes, type 1 diabetes (T1DM) and type 2 diabetes (T2DM), both caused by a combination of genetic predisposition and environmental risk factors:

### 1.3.4 Description of the principals type of diabetes

#### 1.3.4.1 Type 1 diabetes

Type 1 diabetes mellitus (T1DM), or insulin dependent diabetes is an autoimmune disease that occurs when T cells specifically attack and destroy most of the  $\beta$  cells in the pancreas. It appears most often in childhood and involves 10% of patients. Its management requires daily administration of exogenous insulin.

Risk factors related to T1DM [20]:

- Geography: the incidence of T1DM tends to increase when the geographical latitude (distance from the equator) increases. People living in Finland and Sardinia have the highest incidence of T1DM.
- Genetic factors: familial aggregation of T1DM has been recognized for many years, and 10–13% of newly diagnosed children have a first-degree relative affected with type 1 diabetes. Risk of developing islet autoimmunity varies depending on which relative(s) have type 1 diabetes.
- Environmental factors: environmental agents that are suspected to trigger  $\beta$  cell autoimmunity in genetically susceptible individuals include dietary factors and

common viral infections. By today, however, no single factor has been identified that can induce the process of autoimmune  $\beta$  cell destruction.

Type 1 diabetes is associated with the appearance of humoral and cellular islet autoimmunity and a defective immunoregulation in the 90% of type 1 diabetic patients. The disease has different stages starting with a genetic susceptibility then, autoimmunity without clinical disease and subsequent clinical diabetes. First signs of islet autoimmunity appear in early childhood under 10 years of age, with a peak with 2 years of age. Not all children develop the autoantibodies before 2 years, but children with a later development have a slower progression to multiple antibodies. The first antibodies detected are the autoantibodies to insulin (IAAs). After the first response of IAA follows a development of autoantibody to GAD (GADAs) and autoantibody of tyrosine-phosphatase (IA-2 and IA-2 $\beta$ ). Once islet autoantibodies appear, they usually persist. Association of anti-GAD and anti-IA2 is correlated with high affinity in the progression of type 1 diabetes. However, IAA is the least persistent because they may be transferred during pregnancy from the mother with type 1 diabetes. If antibodies are detected in a child early in life, it is important for the assignment of diabetes risk to distinguish whether these antibodies are indeed de novo-produced antibodies of the child or rather antibodies acquired from the mother.

In most children, first peak antibody levels decline and autoantibodies against other  $\beta$  cell antigens may arise sequentially over several years, suggesting regulation and spreading of islet autoimmunity in childhood. Different factors like genes, environment and age are implicated in the progress of the disease [20].

The other 10% of type 1 diabetic patients is due to idiopathic diabetes. The pathophysiologic mechanisms involved in its etiology is unknown, but could be due to glucose desensitization, lipotoxicity, environmental factors, and/or genetic defects in nuclear transcription factors involved in fuel metabolism. Despite the fact that most patients do well for a few years after the diagnosis of diabetes, when treated with diet/oral agents, it seems that insulin therapy maintains lower A1C over the long duration [21].

B cell autoimmunity, marked by the development of islet reactive autoantibodies, portends the development of activated autoreactive T cells capable of destroying  $\beta$  cells, resulting in a progressive and predictable loss in insulin secretory function. Clinical T1D does not present until >80%–90% of the  $\beta$  cells have been destroyed, and there is a marked gap between the onset of autoimmunity and the onset of diabetes. In addition, the degree of  $\beta$  cell destruction required for symptomatic onset is also of growing question, with recent studies suggesting that 40%–50%  $\beta$  cell viability may be present at the onset of hyperglycemia, an aspect that may be related to subject age, among other factors (e.g., body mass index, physical activity, etc.). This may explain why, despite persistent autoimmunity, insulin secretory function can remain stable for long periods of time in persons with T1D. A loss of first-phase insulin response is usually followed by a period of glucose intolerance and a period of clinically “silent” diabetes. Finally, the “slope” reflective of  $\beta$  cell loss in the pre-diabetic period has also recently been subject to considerable debate, with some proposing that the disorder may see its symptomatic onset only following a period of relapsing/remitting like autoimmunity [22].

The symptoms of type 1 diabetes are polyuria (frequent and abundant urination), the polydipsia (thirst), constant hunger, weight loss, impaired vision and fatigue. These symptoms may occur suddenly although the damage to the  $\beta$  cells may begin much earlier and progress slowly and silently [20].

#### 1.3.4.2 Type 2 diabetes

Type 2 diabetes mellitus (T2DM) is one of the most common types of DM, accounted for 90-95% of the diabetic cases worldwide. It is the result of genetic and environmental factors like family history, excessive fat and sugar intake, age and physical inactivity. This diabetes is link with a progressive desensitization of the receptor for the insulin [23, 24].

Glucose is not translocated from the blood stream into the cells because there is a defective regulation of GLUT4 protein due to the inhibition of tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) [25]. In response, pancreatic  $\beta$  cells produce more insulin in order to force the glucose uptake by cells. The increase in insulin production is accompanied by increased islet size and pancreatic proportion of  $\beta$  cells [26]. In presence of hyperinsulinism, the recycle/internalization cycles of GLUT4 can produces a decrease in the number of receptors in the membrane and this effect could be related with the insulin resistance. At this stage, decreased  $\beta$  cells mass is due to apoptosis of  $\beta$  cells mainly caused by glucotoxicity, lipotoxicity, and deposits of islets amyloid polypeptide (IAPP) [27-29].

Pre-diabetes and metabolic syndrome, which follows it, are parts of interrelated common clinical disorders that are accompanied by symptoms of obesity, insulin resistance, glucose intolerance, lipid abnormalities, moderate glycation (A1C 5,7-6,4%), impaired fasting glucose (110-125 mg/dl), and impaired glucose tolerance (140-200 mg/dl). Some studies have reported that approximately 5-10% of pre-diabetic population would suffer from diabetes and problems associated with it in around a year such as heart problems, imbalance in glucose and lipid metabolism, and vascular disorders. Timely interventions in this population can preserve pancreatic  $\beta$  cells, and improve their performance. Current medications to control blood glucose and lipid profile may have dangerous side effects over time such as increased risk of weight gain, liver toxicity, and cardiovascular diseases. Thus, we need to use stronger alternatives with fewer side effects. In this line, nutritional interventions, change in lifestyle, and behavioral therapy are on the rise. However, these interventions may not be effective alone to prevent the development of type 2 diabetes [30].

Although T2DM cannot be cured, it can be treated with a healthy lifestyle such as diet, exercise and weight control which can provide the foundation for managing of T2DM. However, anti-diabetic agents are required to regulate blood glucose levels. These drugs can cause side effects for instance, weight gain which consequently increases the risk of insulin resistance leading to a further enhance in drug dose [31].

#### 1.3.4.3 Other types of diabetes

- **Monogenic diabetes:** Some rare forms of diabetes result from mutations in a single gene and are called monogenic. Monogenic forms of diabetes account for about 1-5% of all cases of diabetes in young people. In most cases of monogenic diabetes, the gene mutation is inherited; in the remaining cases the gene mutation develops spontaneously. Most mutations in monogenic diabetes reduce the body's ability to produce insulin. Neonatal diabetes mellitus (NDM) and maturity-onset diabetes of the young (MODY) are the two main forms of monogenic diabetes. MODY is much more common than NDM. NDM first occurs in newborns and young infants; MODY usually first occurs in children or adolescents but may be mild and not detected until adulthood [32].

- **Secondary diabetes:** It is developed from pancreatic disease, endocrine disease, or administration of certain drugs. When diabetes is secondary to pancreatic disorders, particularly when  $\beta$  cell mass is greatly reduced as in malignancy or pancreatectomy, or when diabetes is due to chemical agents toxic to the  $\beta$  cell, overt diabetes with or without ketoacidosis will often result depending on the extent of  $\beta$  cell loss. In contrast, when diabetes is secondary to endocrinopathies leading to counter regulatory hormone production, overt diabetes or ketoacidosis is unusual, mainly owing to the compensatory responsiveness of the normal  $\beta$  cell mass. The net metabolic outcome in patients with secondary diabetes thus depends on the direct or indirect impact of the underlying disorders on insulin secretion, insulin-sensitivity, and/or unmasking of genetic diabetes [33].
- **Gestational diabetes:** It is developed during pregnancy. It occurs in about 4% of all pregnancies. It is usually diagnosed in the later stages of pregnancy and often occurs in women who have never had diabetes. It is thought to arise because the many changes that occur in the body during pregnancy lead some women to become resistant to insulin or the insulin production is inefficient. There are two reasons why the mother needs more insulin: because during pregnancy some hormones block the insulin action or because the baby increases the mother's need for insulin.

### 1.3.5 Diabetes complications

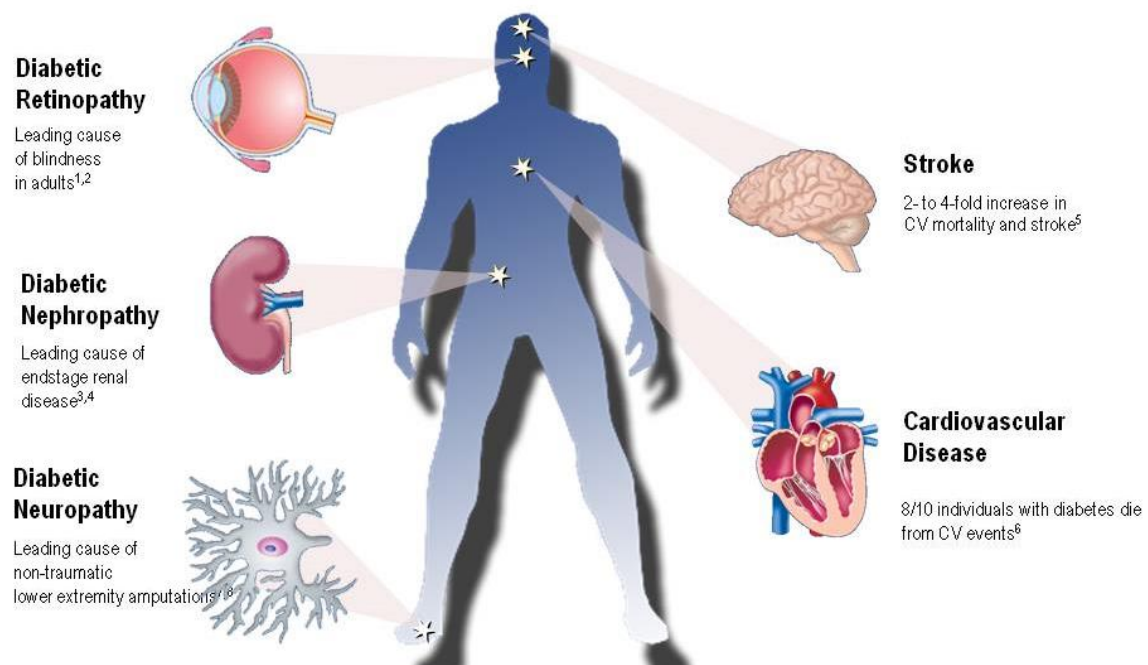
Glycemia fluctuations lead to acute or chronic complications. Acute complications include hypoglycemia diabetic ketoacidosis (DKA), and hyperosmolar hyperglycemic state (HHS):

- **Hypoglycemia** is defined by low blood glucose ( $<4\text{mM}$ ), this may result in a variety of symptoms including clumsiness, trouble talking, confusion, loss of consciousness, seizures, or death.
- **Ketoacidosis (DKA)** is defined by the insulin deficiency with hyperglycemia (glucose levels usually  $>200\text{ mg/dl}$ ) with increased lipolysis, increased ketone production, hyperketonemia and acidosis. Precipitation factor are infection, acute illnesses, lack of diabetes education, poor self-care, inadequate glucose monitoring and indeterminate causes. Morbidity is related to the severity of acid-base and electrolyte disturbances which may result in coma and death [34].
- **Hyperosmolar Hyperglycemic Nonketotic Syndrome (HHNS)** is characterized by the presence of relative insulin deficiency and hyperglycemia, usually  $>600\text{ mg/dl}$  with associated elevated serum osmolality, dehydration, and stupor, progressing to coma correlated with renal failure. Causes of the HHNS are dehydration, medications such as steroids and thiazides, acute illness, cerebral vascular disease and advanced age. The consequences consist of coma and impaired neurologic function with a predisposition to vascular occlusive disease from dehydration or poor perfusion [34].

However, chronic complications can have deleterious impact on numerous tissues including eyes, heart, kidneys, nerves, brain and feet [35]. 60% of the diabetes complications have a cardiovascular disease origin and 30% are from organ degeneration (**Fig. 1-18**):

- **Cardiovascular disease (micro- and macroangiopathy)** are characterized by lesions of the small and large blood vessels of the legs, heart and brain. The elevated blood glucose concentration can produce blood coagulation and increase the blood vessels obstruction near to the heart (heart attack), brain (AVC) or feeds (gangrene).

- **Nephropathy** is refers to the presence of elevated urinary protein excretion in a person with diabetes as a consequence of the high glucose levels which produce an excessive filtration in the kidney. Other factors associated with the development of diabetic nephropathy include diabetes duration, hypertension, hyperglycemia, and smoking. Control of blood glucose and blood pressure reduce the rate of progression of renal disease in diabetes. The presence of albumin is associated with a nearly risk of death from cardiovascular disease.
- **Neuropathy** is the result of damage of the peripheral or autonomic nerves, produced by prolonged exposure to high blood glucose. It can result in traumatic injuries, infections, metabolic problems, inherited causes, exposure to toxins and urinary tract infections.
- **Retinopathy** is characterized by alterations in the small blood vessels in the retina. High blood pressure, early age at onset of diabetes and longer duration of diabetes are associated with increased risk of progression of retinopathy. Causes of decreased vision in patients with diabetes are cataract (clouding of the lens), glaucoma (damage to the optic nerve), and corneal disease.



**Figure 1-18 Diabetes complications**

Source: Sanofi

## 1.4 Diabetes treatments

### 1.4.1 Oral antidiabetic drugs (OAD) for type 2

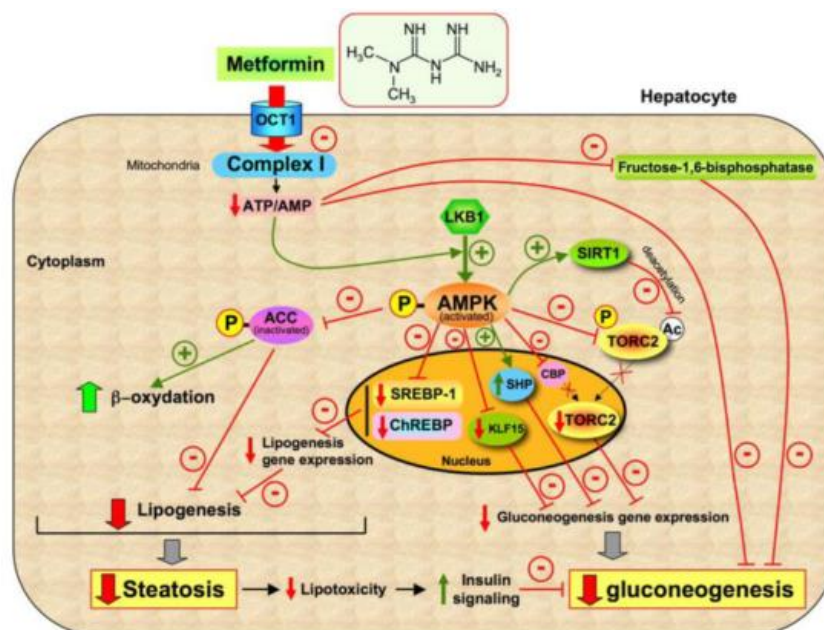
Antidiabetic agents aim to achieve normoglycemia and relieve diabetes symptoms, such as thirst, polyuria, weight loss and ketoacidosis in type 2 diabetic patients. The long term goals are to prevent the development or slow the progression of long term complications of the disease.



### 1.4.1.1 Metformin

Metformin, a biguanide that acts directly against insulin resistance, is regarded as an insulin sensitizing drug. Available formulations include Glucophage<sup>®</sup>, Glucophage XR<sup>®</sup>, Riomet<sup>®</sup>, Fortamet<sup>®</sup>, Glumetza<sup>®</sup>, Obimet<sup>®</sup>, Dianben<sup>®</sup>, Diabex<sup>®</sup> and Diaformin<sup>®</sup>. Because of its safety and efficacy, metformin is the cornerstone of monotherapy, and joint guidelines recommend that metformin be initiated as first line monotherapy unless a contraindication such as renal disease, hepatic disease, gastrointestinal intolerance or risk of lactic acidosis coexists [36].

Despite being the most widely used OAD in the world, metformin can reach a plateau of effectiveness due to progressive  $\beta$  cell failure. Metformin is only effective when there is sufficient endogenous or exogenous insulin and, because of this, patients are unable to maintain tight glycemic control as their disease progresses [37, 38] (**Fig. 1-18**).



**Figure 1-19 Action mechanism of metformin [39]**

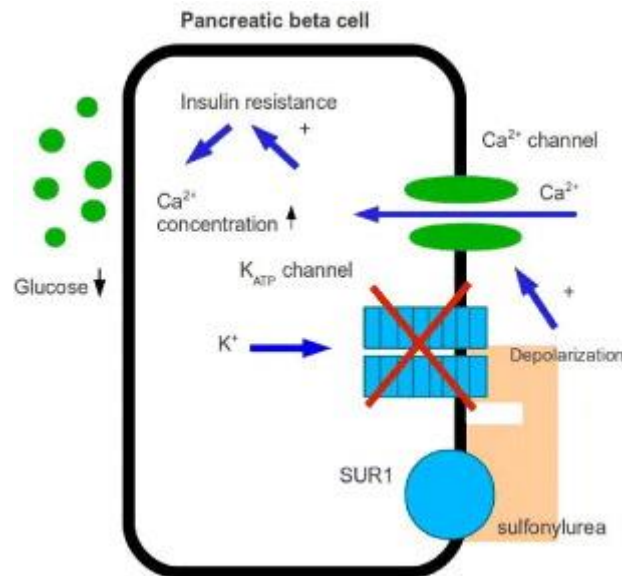
### 1.4.1.2 Insulin secretagogues

These main classes include agents that stimulate insulin secretion in  $\beta$  cells through specific receptors (sulphonylureas and glinides) or agents that increase GLP-1, insulin secretagogue hormone from the pancreas. The GLP-1 increase could be related with a reduction of the endogenous GLP-1 destruction by DPP4 (DPP4 inhibitors and gliptins) or with a GLP-1 resistance to DPP4 (GLP-1 analogs and GLP-1 agonist receptors).

#### 1.4.1.2.1 Sulphonylureas

Sulphonylureas (SUs) are the oldest and most widely used medications for the treatment of T2DM. Although SU therapy effectively lowers blood glucose concentrations (average decrease in FPG of 2-4 mmol/l, accompanied by a decrease in A1C of 1-2%) by stimulating insulin secretion from  $\beta$  cells, treatment with SUs is associated with a progressive linear decline in  $\beta$  cell function. Eventual inability to maintain glycemic control reflects an advanced stage of  $\beta$  cell failure. Hypoglycemia is the most common and most serious adverse event

associated with SU therapy, mainly because of insulin release being initiated even when glucose concentrations are below the normal threshold for normal physiologic glucose-stimulated insulin release. Weight gain, regarded as a class effect of SUs, is thought to result from an anabolic effect of increased insulin concentration. Owing to decreased effectiveness of SUs over time and an associated decline in the insulin secretory reserve, combination therapy has focused mainly on adding insulin-sensitizing medications, including metformin and thiazolidinediones [40-42] (**Fig. 1-20**).



**Figure 1-20 Action mechanism of sulfonylureas**  
Source: <http://typetwodiabetes.info>

#### 1.4.1.2.2 Glinides

Meglitinides such as repaglinide and nateglinide are prandial insulin releasers that stimulate rapid insulin secretion. Repaglinide (NovoNorm<sup>®</sup>, Prandin<sup>®</sup>, GlucoNorm<sup>®</sup>) is the first clinically available insulin secretagogue that specifically enhances early-phase prandial insulin response by increasing the sensitivity of  $\beta$  cells to elevated glucose levels, producing a greater insulin release under hyperglycemic conditions. Rapid-acting insulin releasers can be suitable for lifestyles where meals are unpredictable or missed. Lower risk of hypoglycemia makes these agents an attractive option for some elderly patients, in particular when other agents may be contraindicated [43, 44].

#### 1.4.1.2.3 Dipeptidyl Peptidase-IV inhibitors: gliptins

Dipeptidyl peptidase-IV (DPP-IV) inhibitors such as gliptins, suppress the degradation of a variety of bioactive peptides, including glucagon-like peptide-1 (GLP-1), leading to an enhancement of their action. GLP-1 inhibits glucagon release, which in turn increases insulin secretion and decreases blood glucose levels. DPP-IV inhibitors are orally administered drugs with a significant effect on glucose tolerance and lasting improvement of A1C. DPP-IV inhibitors are weight-neutral and well tolerated [45].

#### 1.4.1.2.4 GLP-1 analogs or GLP-1 receptor agonist

GLP-1 analogs have therefore been developed to produce a longer terminal elimination half-life, and these have been shown to cause weight loss and improvement in

glycemia in the diabetic population. A meta-analysis of GLP-1 analogues showed that these were able to reduce A1C by 0.97%. It has been postulated that shorter acting GLP-1 analogues (exenatide and lixisenatide) reduce hyperglycemia primarily by slowing gastric emptying, whereas longer acting GLP-1 analogs (liraglutide, exenatide long-acting release, albiglutide and dulaglutide) predominantly lower postprandial glucose levels by insulinotropy and glucagon inhibition [46].

#### 1.4.1.3 SGLT2 inhibitors

Sodium-glucose co-transporter 2 (SGLT2) inhibitors are a new class of diabetic medications. In conjunction with exercise and a healthy diet, they can improve glycemic control. They have been studied alone and with other medications including metformin, sulfonylureas, pioglitazone, and insulin. Inhibition of SGLT2 leads to the decrease in blood glucose due to the increase in renal glucose excretion. The mechanism of action of this new class of drugs also offers further glucose control by allowing increased insulin sensitivity and uptake of glucose in the muscle cells, decreased gluconeogenesis and improved first phase insulin release from the  $\beta$  cells.

#### 1.4.1.4 $\alpha$ -glucosidase inhibitors

$\alpha$ -glucosidase inhibitors, including acarbose, are competitive inhibitors of membrane-bound intestinal  $\alpha$ -glucosidases that hydrolyze oligosaccharides, trisaccharides and disaccharides to glucose and other monosaccharides in the small intestine and thereby delay postprandial glucose absorption. These agents are available as a first-line treatment in patients with slightly raised basal glucose concentrations and marked postprandial hyperglycemia (average decrease in A1C of 0.5–1%). The use of  $\alpha$ -glucosidase inhibitors in combination with sulfonylureas, metformin or insulin can improve glycemic control. Despite their good [42, 47].

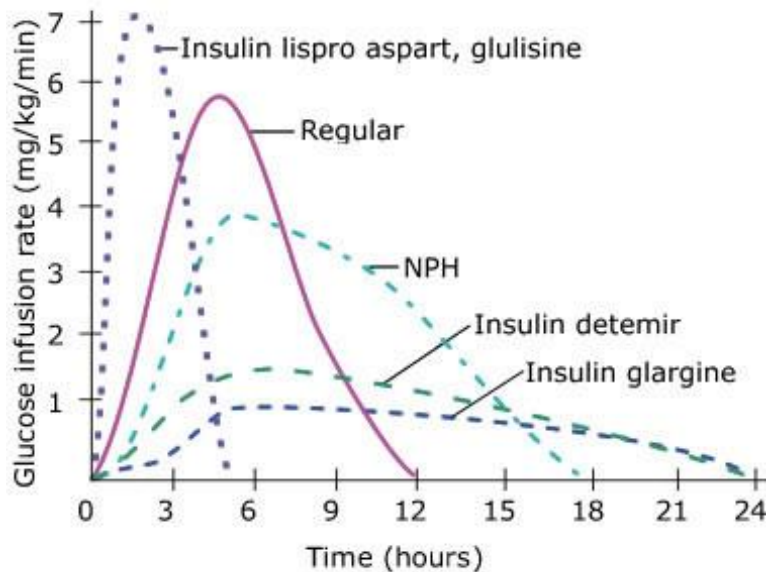
### 1.4.2 Insulin therapy

Insulin administration is performed in type 1 diabetic as first treatment and in type 2 diabetes patients for whom oral antidiabetic drug therapy become inefficient. Insulin is usually administered subcutaneously using a syringe, insulin pen or insulin pump. Which insulin regimen is best for patient depends on factors such as the type of diabetes, blood sugar fluctuation throughout the day and lifestyle.

It is important where insulin is injected in the body, because it affects the blood glucose level. Depends of injection site, insulin goes into the blood with different speeds. For this reason, it is preferable to inject insulin always in the same site. The fastest site is in the abdomen and from the upper arms is slower [45].

Each insulin type is characterized by the starting time (onset), maximal efficiency (peak) and the lifespan (duration) (**Fig. 1-21**):

## Activity Profiles of Different Types of Insulin



**Figure 1-21 Insulin characterization in function of the onset, peak and duration**

Source: University of California, San Francisco, UCSF

### 1.4.2.1 Fast-acting human insulin

Synthetic human insulin administered by all routes (intravenous, intramuscular, subcutaneous and intraperitoneal). The onset of action depends of the administration route. The effect when is injected subcutaneously starts 15-20 minutes after injection, reach the peak between 2 and 4 hours and finish after 6 hours. It is presents in a clear solution that can be used at all times in all injection systems: syringes, pens and pumps. However, it is less stable than ultrafast analogs in external pumps. In common use, it is administered subcutaneously in single or multiple daily injections usually associated with other long-acting insulin injections.

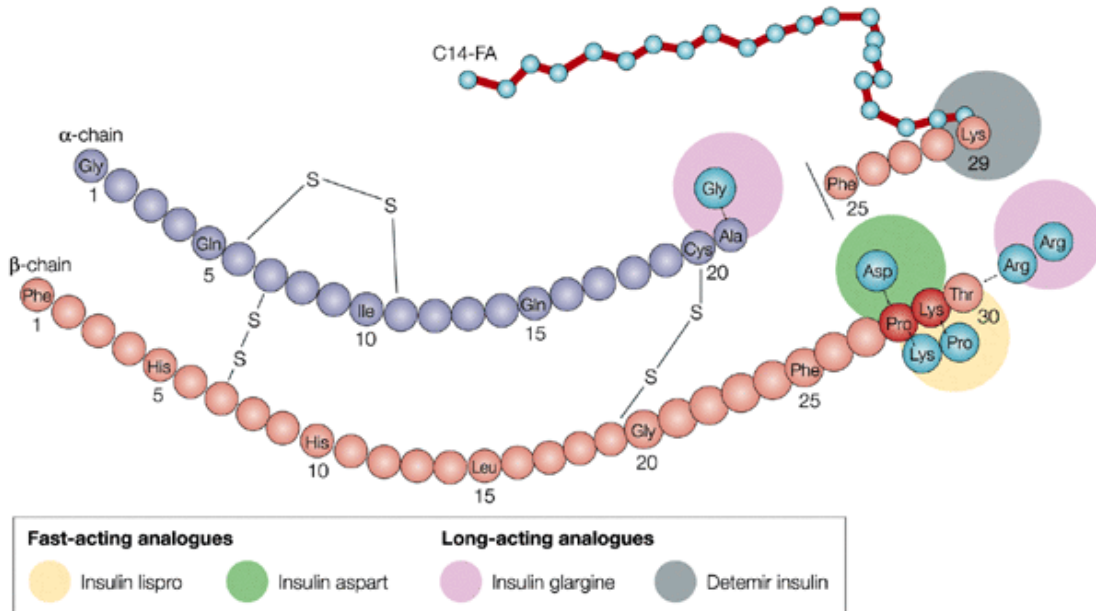
### 1.4.2.2 Insulin NPH

NPH (Neutral Protamine Hagedorn) is an intermediate-acting biogenetic human insulin with a protamine sulfate added, for delaying and increasing its duration of action. This insulin is still widely used under the names Umuline<sup>®</sup> NPH (Eli Lilly) or Insulatard<sup>®</sup> (Novo Nordisk) as intermediate-acting insulin. It is in the form of an injectable suspension in vial or pen. The duration of action of NPH insulin continues for 14 to 18 hours. This period is called intermediate because it is between the fast-acting insulin and the long-acting insulin.

### 1.4.2.3 Insulin analogs

An insulin analogue is a modified form of human insulin but still functional on the patient to perform the same action than human insulin in terms of glycemic control. By genetic engineering, the amino acids of the insulin can be changed to modify its absorption characteristics, distribution, metabolism and excretion (**Fig. 1-22**). These modifications have been used to create two types of insulin analogues:

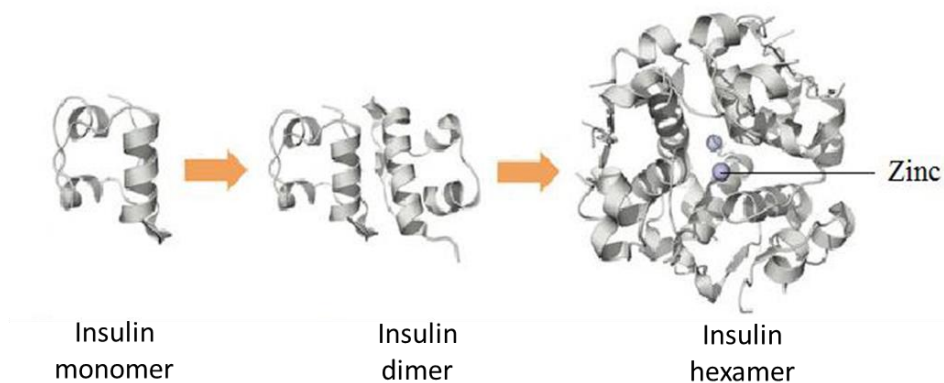
- Those which are slowly released over a period between 8 and 24 hours. They are used to maintain basal insulin throughout the day.
- Those that are more readily absorbed from the injection site and thus act more quickly. They are intended for use with prandial bolus.



**Figure 1-22 Modification sites in the insulin sequence**  
Source: Nature Reviews, Drug Discovery

#### 1.4.2.3.1 Long-acting injected insulin analogs

Long-acting insulin analogues can be obtained by modifying the spatial conformation of insulin. Indeed, in the presence of zinc, insulin forms a hexamer: three dimers set around a threefold axis passing through 3 zinc atoms. So, their diffusion is slowed, which contributes to increase the duration of action of insulin (**Fig. 1-23**).



**Figure 1-23 Insulin oligomerization**

These long-acting analogues arise from modification of the structure of human insulin, but this modification is intended to increase their duration of action. Their release is performed in a constant way reducing the risk of hypoglycemia. There are two long-acting analogs:

- Insulin glargine or Lantus<sup>®</sup> (Sanofi-Aventis) is soluble at pH 4 and is in the form of a clear solution. Once injected into the subcutaneous tissue, it precipitates in the microcrystals allowing its gradual release. Its action begins within 2 to 4 hours and extends regularly and consistently without peak until 25 hours. This kinetic allows the one daily injection at a fixed time.
- Insulin detemir or Levemir<sup>®</sup> (NovoNordisk) is in the form of a clear neutral solution. It binds in the subcutaneous tissue which gives it its delay effect. It begins to work 2 to 4 hours after injection. Its action continues without peak until 14 hours after injection.

#### 1.4.2.3.2 Rapid-acting injected insulin analogs

They are injected subcutaneously and insulin action begins to act more quickly than soluble human insulin within 10 minutes after injection. They maintain lower blood glucose within 4 hours after the meal, with a maximum effect occurring 1-3 hours after injection. Their action duration is lower than soluble human insulin and there is a rise in blood glucose meals distances beyond 4 hours after injection. These analogs generally replace fast insulin in emergency treatment and in multiple injection regimes. They are administered immediately before the meal so that the human insulin should be 30 minutes before. They allow better reduce postprandial glucose elevations and avoid episodes of hypoglycemia between meals. Ultrafast analogs are perfectly stable in the external pumps but not in implantable pumps.

There are three fast analogs (**Fig. 1-22**):

- that resulting from the reversal of the two penultimate amino acids on the  $\beta$  chain, taking the lysine the  $\beta$ 28 position and the proline the  $\beta$ 29 position to give insulin lispro or Humalog sell by Eli Lilly,
- that obtained by replacing proline in position  $\beta$ 28 by an aspartate to give insulin aspart or Novorapid developed by the laboratory Novo Nordisk,
- that replacing the aspartate in position  $\beta$ 3 by the lysine and the lysine in position  $\beta$ 29 with a glutamate to give insulin glulisine or Apidra (Sanofi-Aventis).

#### 1.4.2.4 Insulin administration devices

##### 1.4.2.4.1 Insulin pens

Insulin dose is dialed on the pen, and is injected through a needle, much like using a syringe. Cartridges and pre-filled insulin pens only contain one type of insulin. Two injections must be given with an insulin pen if using two types of insulin. Those pens are light, easy to transport and discreet. However, the repeated injections can be painful and it can produce lipoatrophy, lipohypertrophy or allergic reactions (**Fig. 1-24**) [35].



**Figure 1-24 Insulin pen**

#### 1.4.2.4.2 Insulin pumps

Insulin pumps (**Fig. 1-25**) are small computerized devices that deliver insulin in a continuous and regulable way. Doses are delivered through a flexible plastic tube called a catheter. With the aid of a small needle, the catheter is inserted through the skin into the fatty tissue and is taped in place. Pumps can be programmed to release small doses of insulin continuously (basal), or a bolus dose close to mealtime to control the rise in blood glucose after a meal. This delivery system most closely mimics the body's normal release of insulin.



**Figure 1-25 Insulin pump**

#### 1.4.2.4.3 Artificial pancreas

The artificial pancreas (AP), known as closed-loop control of blood glucose in diabetes, is a system combining a glucose sensor, a control algorithm, and an insulin infusion device. Although the intravenous route of glucose sensing and insulin infusion is unsuitable for outpatient use, these devices proved the feasibility of external glucose control and stimulated further technology development. The new wave of control designs, model-predictive control (MPC), is based on prediction of glucose dynamics using a model of the patient metabolic system and, as a result, appears better suited for mitigation of time delays due to subcutaneous glucose sensing and insulin infusion. In addition, MPC is a better platform for incorporation of predictions of the effects of meals and for introduction of constraints on insulin delivery rate and glucose values that safeguard against insulin overdose or extreme blood glucose fluctuations [48].

### 1.4.3 Transplantation

In some cases, usually after several decades of diabetes, insulin therapy becomes inefficient and complications like neuropathy prevent the detection of hypoglycemia which can lead to coma. Hypoglycemia unawareness, renal failure and lifestyle degradation with loss of independence can lead the practitioner to propose transplantation to restore a physiological regulation of glycemia and improve the general state of the patient [49]. Pancreas or islet transplantation alone or with kidney transplantation is usually performed after a close look on the beneficence/risk balance. Indeed, the invasive surgery for pancreas transplantation and the establishment of immune suppressive regimen for both have potential

detrimental effects, therefore only the patient with life threatening complication can be included in the recipient waiting list.

Most of the diabetic patients become transplant candidates after 20-25 years of insulin therapy failed. By this time, some of these patients have developed already diabetic nephropathy or renal failure and they receive a simultaneous pancreas and kidney transplant (SPK) or islet after kidney transplantation (IAK). Other possibility depending on the organ availability is kidney transplant first and then pancreas transplant to reverse diabetes (PAK) [50]. Simultaneous islet-kidney transplantation (SIK) may also be considered, but is often not feasible because of the challenges involved in islet isolation, which may fail to yield sufficient islets for transplantation [51].

Selected diabetic patients may also be considered for pancreas transplantation alone (PTA) or islet transplantation alone (ITA) when renal function is normal. PTA and ITA improves the course of diabetic retinopathy [52], diabetic neuropathy [53], and diabetic nephropathy [53-55], and reduces the level of cardiovascular risk [53, 56].

#### 1.4.3.1 Pancreas transplantation

Transplantation of the pancreas is one of the most effective strategies to cure type 1 diabetes. It is expected to restore insulin independence in diabetic patients but long immunosuppression and hard surgery are the principal limit factors to have successful pancreas transplantation.

During a pancreas transplant, the recipient's diseased pancreas is left in place. The donated pancreas is placed in the front part of the abdomen and connected to the lower abdominal blood vessels. The donated duodenum is attached to either the recipient's intestine or bladder so that pancreatic secretions can drain.

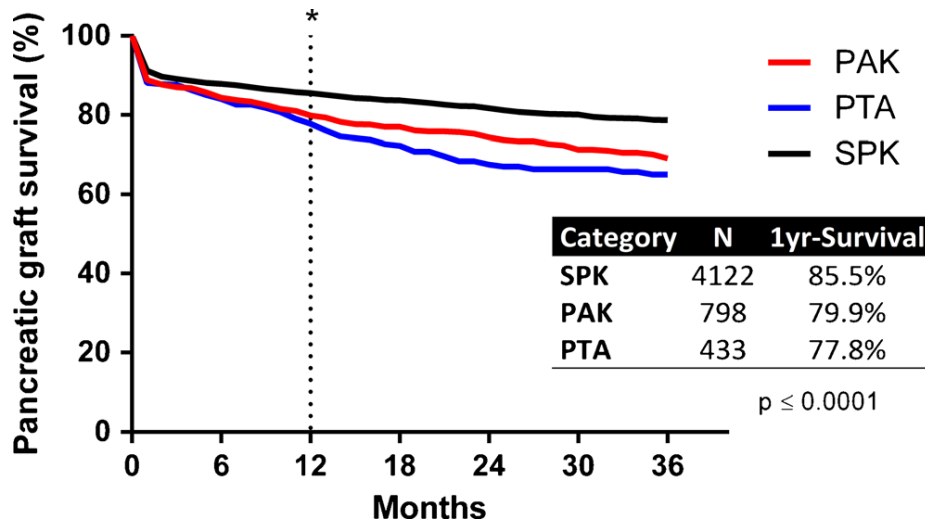
Risks associated with pancreas transplantation include clinical complications caused by the surgery and by chronic immunosuppressive drugs, as well as death. Perioperative complications leading to relaparotomy occur in 30% of patients and include intra-abdominal infections and abscess, vascular graft thrombosis, an anastomotic leak, and duodenal stump leak. Most of those complications are related with the donated pancreas. Drug related complications include bacterial and viral infections (particularly cytomegalovirus) and malignancy (particularly skin tumors and lymphoma) secondary to chronic immunosuppression. Other drug related complications include osteoporosis and insulin resistance (steroids) and decreased renal and pancreatic cell function (cyclosporin, tacrolimus) [49]. The major causes of death after transplant surgery are cardiovascular and/or cerebrovascular problems and infections [57].

Today, the immunosuppressive drug used by many centers is a combination between calcineurin inhibitors (CNIs) like tacrolimus, and inhibitors of the target of Rapamycin (mTORI), such as sirolimus and everolimus, provided an opportunity to reduce both the diabetogenic and nephrotoxic potential of the immunosuppression [53].

More than 40,000 pancreas transplants have been performed worldwide, and patient survival rates have improved significantly over time in all categories of pancreas transplantation [58]. According to the International Pancreas Transplant Registry, the patient survival rates are >96% at 1 year post-transplant and >80% at 5 years. In some single center experiences, high actual patient survival at 10 years has been reported in both SPK and PTA [53, 59].



Pancreas graft survival rate is based on insulin independence. In the past decade, unadjusted graft survival rates at 1 year were 85% (SPK), 80% (PAK) and 77% (PTA). The rates at 5 years were 71% (SPK), 65% (PAK) and 58% (PTA). More recently, [53, 59] 10 yr actual insulin independence rates have been reported to be >80% in SPK and >60% in PTA (**Fig. 1-26**) [59].



**Figure 1-26 Graft survival among adult pancreas transplant recipients in the US from 2005 – 2010. All transplants are from deceased donors registered by IPTR/UNOS [51]**

The estimated half-life in pancreas graft is now 14 years (SKP), 7 years (PAK) and 7 years (PTA). Moreover, the estimated half-life has increased to 10 years in recipients of PAK or PTA with a functioning pancreas graft at 1 year post-transplant [50].

Rejection is the main cause of pancreas loss [60, 61]. Autoimmunity is also increasingly recognized as a cause of cell failure. Hyperglycemia occurs only in cases of severe cell dysfunction or destruction, and therefore it is a late marker of rejection [62]. Among the different causes of graft loss, recent studies have proven that despite immunosuppression, the recurrence of autoimmune disease is not a rare event [63]. Immunosuppression usually prevents such recurrence, but not in all patients [63-67].

Although pancreas transplantation has a higher success rate, it is usually associated with higher morbidity due to surgical complications, urinary tract infections, acute postoperative hematuria, sterile cystitis, urethritis and balanitis, metabolic acidosis, reflux pancreatitis, urine leak and intra-abdominal abscess. Therefore, in some places, the strategy of pancreas transplantation for type 1 diabetic patients has been withdrawn. That is why islet transplantation rise interest since this cell therapy triggers less morbidity [68].

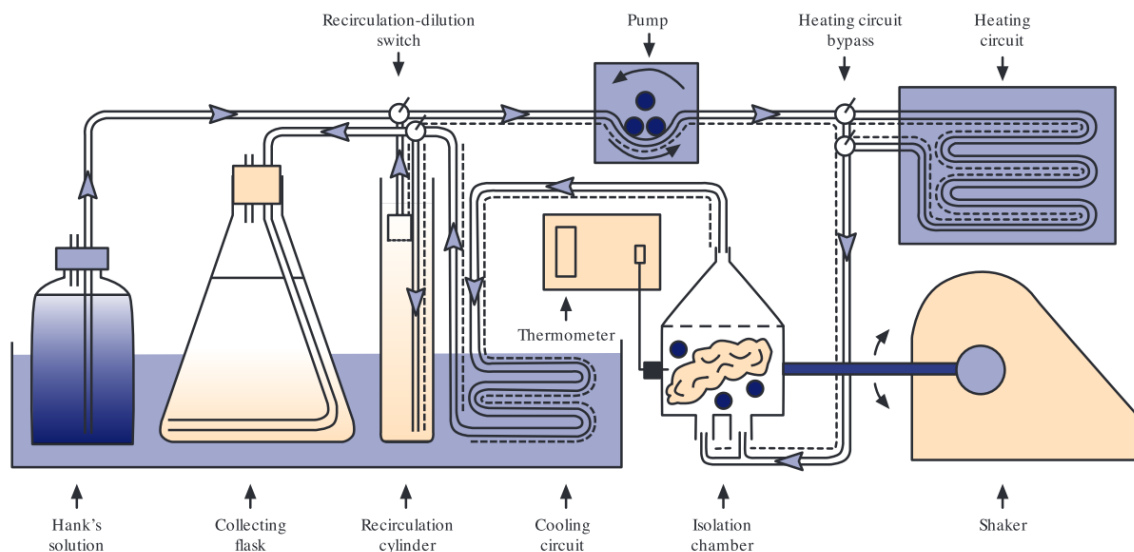
#### 1.4.3.2 Islet transplantation

In type 1 diabetes, the exocrine function of the pancreas remains intact. Therefore there is no need of transplanting this tissue. The only tissue require to restore glycemic control is  $\beta$  cells. However, the function of  $\beta$  cells is under the control of other cells in the pancreatic

islets, therefore to insure a proper and the fine regulation of glycemia, whole islet needs to be transplanted. The requirement challenges the therapy, since islets are considered as mini organs which make more complex the production of viable and functional islets. Only primary culture can generate islet today. Islet transplantation as cell therapy has been developed for the last 30 years.

#### 1.4.3.2.1 Story

Successful pancreatic islet transplantation became a reality for humans in 1980. Since then, the protocol has been optimized and the only option to get islets is to isolate them from the whole pancreas using an enzymatic digestion by collagenase. The first who used the enzyme collagenase to isolate viable islets from guinea pig pancreas was Moskalewski in 1965 [69]. In 1967, Lacy and Kostianovsky improved this technique by intraductal distension of the pancreas prior to digestion, by employing sucrose gradients for centrifugal separation of the islets from the pancreatic digest [70]. In 1984, Gray and colleagues at Oxford successfully adapted the canine methodology to human pancreas [71] and in 1988, Camilo Ricordi developed a semi-automatic method of human islets isolation (**Fig. 1-27**) and at that time all the clinical trials started [72]. This isolation technique is the base of the actual protocols. The procedure starts with the collagenase injection through the pancreatic duct in the donor pancreas, the pancreas is cut into pieces and place in the digestion chamber (Ricordi chamber) connected to a close loop of medium heat up to 34°C, to activate the enzyme. Once islets are released from the tissue, which control by biopsies and observation under the microscope, the circuit is opened to collect the islets and stop the enzymatic reaction. Indeed, islets contain collagen in their structure. Therefore they could be dismantled if there are in contact too long with the collagenase. The digest obtained contained exocrine and free islets that need to be separated to get pure islets. This step is called purification. The purification consists in the separation of islets from the rest using a density gradient. The density of an islet is lower than exocrine, ducts... the gradient has been established to fit with the islet density and the rest pellets at the bottom of the gradient [73].

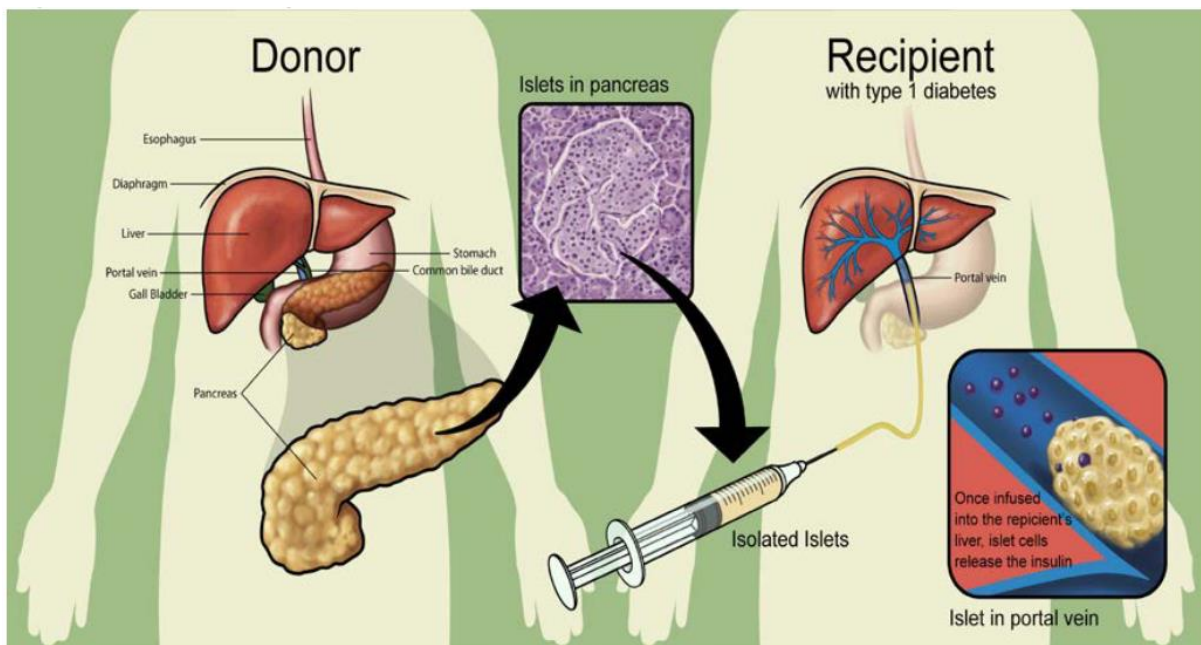


**Figure 1-27 Semi-automated human islet isolation procedure [73]**

Between 1977 and 1980, ten patients with chronic pancreatitis received intraportal transplantation of autologous islet tissue after pancreatectomy. One patient died of a complication not related to the islet autotransplant and of the nine remaining patients; four had been insulin independent for 1, 9, 15, and 38 months. Three decreased their insulin requirement to 15-30 units per year and two patients 35 and 60 units of insulin per day [74]. In 1992, Pyzdrowski reported that 265,000 islets were sufficient to achieve insulin independence [75].

In 2000 a big step was done. For the first time, Shapiro and colleagues performed islet transplantation in 7 patients with type 1 diabetic and insulin-independence was achieved with normoglycemia during one year [76]. The major breakthrough was the corticoid free immunosuppressive regimen. After these results, this protocol has been called Edmonton protocol and set up in many centers. Edmonton protocol components are:

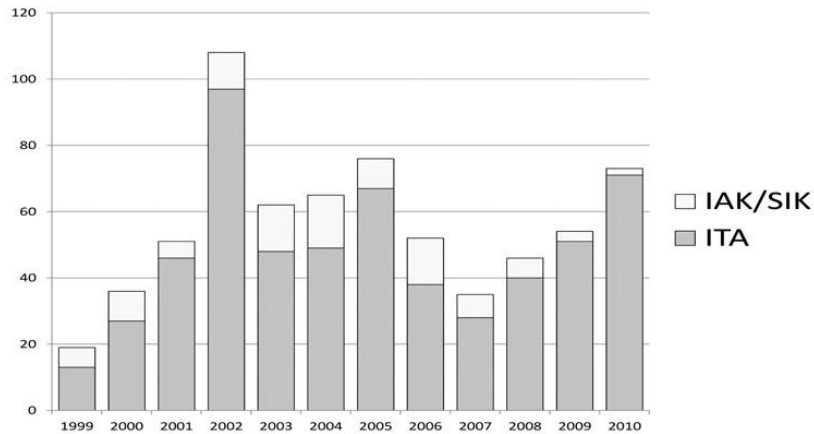
- Selection of type 1 diabetes patient with severe hypoglycemia or unstable diabetes.
- Immunosuppressive treatment with dacluzimab (anti-CD25mAb) mixed with sirolimus (mammalian Target of Rapamycin inhibitor, mTOR) and tacrolimus (Calcineurin inhibitor).
- Transplanted islets are prepared in absence of xenogeneic protein (bovine albumin is replaced by human albumin).
- Insulin therapy is not interrupted immediately after transplantation in order to promote the graft organization and implantation.
- Islet infusion is performed by the portal vein with local anesthesia and under ultrasonography control.
- In the transplantation, 10,000 islets equivalents (IEQ) are transplanted per kilogram of the patient body weight. Islets infusion is performed just few hours after isolation (**Fig. 1-28**).



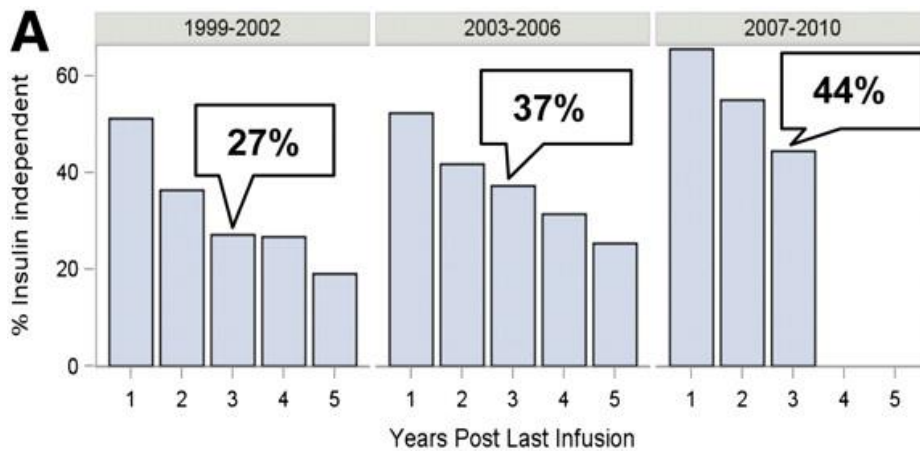
**Figure 1-28 Pancreatic islets transplantation [77]**

Since 2000, 677 islet transplants alone or after kidney have been done all over the world, using new advances and protocol changes (**Fig. 1-29**). Insulin independent at 3 years after

transplant improved from 27% in the early area (1999-2002) to 37% in the mid (2003-2006) and to 44% in the most recent area (2007-2010) (**Fig. 1-30**) [78]. Despite those encouraging results, the therapy remains confidential because of several limiting factors. The requirement of two to three pancreases to get enough islets for the reversion of diabetes (10,000IEQ/kg patient) is the major restriction of islet transplantation due to the lack of donors.



**Figure 1-29** Islet allograft recipient (n=677) registered in the Collaborative Islet Transplant Registry (CITR) according to type of transplant (n per year) [78]



**Figure 1-30** Rates of insulin independence after allogeneic islet infusion (islet transplant alone and IAK), annually after last infusion [78]

The use of immunosuppressive treatment during all the patient life, the lack of donors and the inefficiency of the isolation technique are the major problems to use this therapy as an insulin therapy for all diabetic patients.

#### 1.4.3.2.2 Islet transplantation procedure

##### 1.4.3.2.2.1 Donor selection

Different factors like the donor age, body mass index (BMI), ischemia-reperfusion time and surgical team are really important to obtain successful islet isolation. Long cold

ischemia time, donor hyperglycemia and donor cardiac arrest were associated with poor islet yield. Uncontrolled hyperglycemia in donors (blood glucose levels of >15mmol/l) is related with reduced isolation success. Ischemia reperfusion is another factor to consider, if the pancreas is preserved in cold solution more than 6 hours and the donor cardiac arrest is more than 5 min, islet isolation success could be reduced [79]. The age of the donor have an impact on the quality of the purification. Islets from pediatric donors are difficult to separate from the exocrine tissue without fragmentation. The structure of the extracellular matrix is different with age, therefore the collagenase, specific for one particular collagen, does not have the same efficiency. Islet yields from young (2-15 years old) and old (56-69 years old) donors compared with adult donors (18-55 years old) are reduced. Also, increased in body mass index is related with higher recovery of islets and increased donor body surface area is associated with pancreatic insulin content. The appearance of the pancreas is also important for isolation success, if the pancreas is damage the success will be reduced [79].

#### 1.4.3.2.2.2 Recipient selection

To select the good candidate for islets transplantation there is a drastic selection associated with a benefit/risk analysis. Islets transplantation procedure and immunosuppressive treatments are the main risk factors. In fact, the transplantation procedure can generate risk of bleeding, thrombosis, biliary perforation, elevation of the serum transaminase and arteriovenous fistula. In the other hand, immunosuppression treatment can produce mouth ulcers, diarrhea, anemia, loss of weight, fatigue, elevation of low density lipoproteins, hypertension, kidney failure and peripheral edemas [80].

Inclusion factors for islet transplantation are mainly for patient with unstable type 1 diabetes. Islet transplantation is proposed to type 1 diabetic patients with kidney function stable but associated with a persistent metabolic instability due to the glycemic lability index and/or non-perception with pronounced hypoglycemia despite all the possible efforts to optimize the glycemia control [80] (**Table. 1-2**).

INCLUSION CRITERIA	EXCLUSION CRITERIA
<p><b>Type 1 Diabetes for greater than 5 years:</b> Negative / negligible C peptide (fasting and/or stimulated)</p>	<p><b>BMI greater than 30</b> (will consider BMI greater than 30 on individual basis)</p>
<p><b>Age greater than 20 years</b></p>	<p><b>Current or recent smokers</b> (more than zero cigarettes at any time in previous 6 months)</p>
<p><b>Retinopathy:</b> all stages</p>	<p><b>Planned pregnancy</b></p>
<p><b>Renal Status:</b> <b>A) little or no evidence of significant diabetes related renal disease.</b> - Creatinine clearance greater than 70 mL/min/1.73m (GFR or Nuclear). - Documented history of albumin/creatinine ratio greater than 1.8 mg/mmol in men and 2.5 mg/mmol in women <b>B) severe hypoglycemic unawareness</b></p>	<p><b>Malignant hypertension</b> causing end stage organ damage (retinopathy, stroke, acute coronary syndrome) <b>Severe concurrent illness</b> likely to limit life or require extensive systemic treatment <b>Active infection or evidence of ongoing or recurrent viral disease</b></p>
	<p><b>Inadequate understanding, compliance, or unwillingness to participate with clinical requirements of the Islet transplant program</b></p>

**Table 1-2 Criteria for recipient inclusion or exclusion in islets transplantation [80]**

#### 1.4.3.2.2.3 Pancreas preservation

After retrieval, the pancreas is placed in preservation solution. Once the organ is cut from the general circulation it is cooldown rapidly and ischemia starts. During cold ischemia the metabolism is slowdown and degradation of ATP in ADP, AMP and adenosine occurred. The production of adenosine, inosine and hypoxanthine are continued during the organ preservation. These metabolites are able to cross the cellular membrane and are susceptible to be eliminated during the storage and reperfusion process [81]. The elimination of these metabolites limits the cell capacity to regenerate ATP.

It is important to identify the potential risks in the preservation and conservation of the organ. The principal factors are the temperature, organ rinse, ischemia-reperfusion time and the physical, chemical or pharmacological effects of the conservation solutions [82].

University of Wisconsin solution (UWS) has been the standard preservation solution for pancreas transplantation for almost 20 years. Recently, multiple reports have suggested that other preservation solutions as histidine-tryptophan-ketoglutarate (HTK) may be effective alternatives to UWS. Different studies comparing UWS and HTK have been performed. One of them showed 77 consecutive pancreas recipient, 41 in UWS and 36 in HTK with no

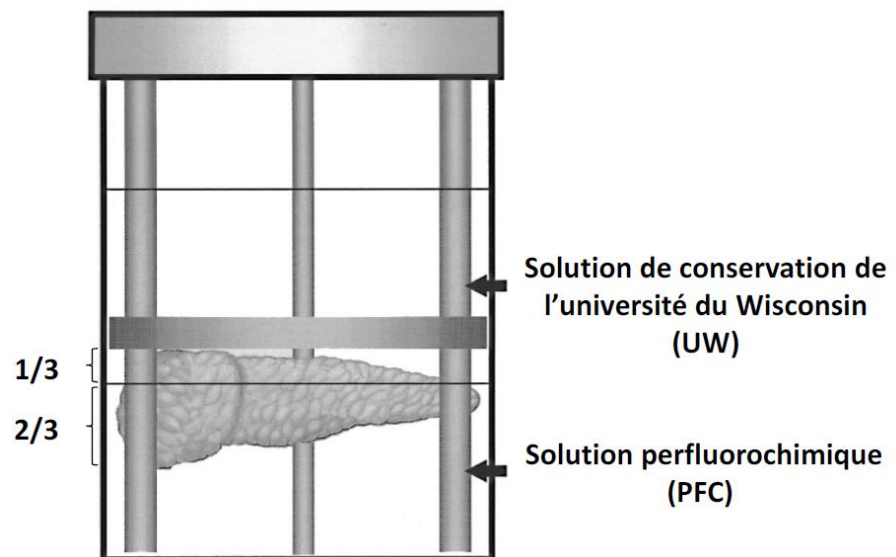
significant difference in graft function after 90 days post-transplantation. Furthermore, islet yields from human pancreases preserved in UWS or HTK were equivalent [83].

Moreover, since the pancreas is preserved at 4°C, the ATP synthesis is limited by the adenine nucleotide translocator enzyme which is sensible to the temperature. This enzyme is linked to the membrane, which transport the ADP into the mitochondria. More energy is needed for enzyme activation below 18°C, and is reflected in the membrane fluidity changes induced by the cooling of the lipids in the membrane [84]. Moreover, the production of reactive oxygen species, which is one of the main causes of pancreatic  $\beta$  cell dysfunction, is also related with a decrease in ATP production [85]. So far, supply in adenosine could be important for  $\beta$  cell survival since ATP is implicated in islets function, insulin secretion [80] and in protection against oxidative stress [81].

Previous studies showed that supply in adenosine help to reformation of the pool of ATP and the combination of adenosine and perfluorochemical oxygen carriers (PFCs) during the process of pancreas preservation improved the levels of ATP [86, 87]. Adenosine contained in UWS prevents a graft edema providing the substrates for the metabolism and prevents the deterioration of the peri-insular extracellular matrix [88].

- Two layer method (TLM):

This method was used for pancreas preservation, which are for islet isolation in animal models [89]. The TLM uses UWS mixed with PFC. The pancreas is between both immiscible layers consists in (**Fig. 1-31**):



**Figure 1-31 Two layer method for pancreas preservation [90]**

Fluor (F) is the element more electronegative in the periodic table that means there is a high affinity for the electrons which confers to the fluoro derivatives a good stability. The link between F and the other elements is really strong, especially the link F-C ( $E=552\text{kJ}\cdot\text{mol}^{-1}$ ). PFCs are chemically inert and in the aqueous medium they are insoluble. As no hydrogen present in the molecule, no hydrogen bonds are existing providing to PFCs hydrophobic and lyophobic characteristics [91].

Because of this absence of hydrogen bonds, molecules which composed PFCs are sitting there side by side with no interaction which leaves space in between, space that can be filled with gases, that is why PFCs have high gas dissolving potential. Due to all this stability and gas solubilization properties, PFCs are exceptional blood artificial substitutes and are used in the blood transfusions and in the organ preservation before transplantation for oxygenation [92, 93].

TLM has been shown until 2007 to improve the yield, viability, morphology, isolation achievement, transplantation and islets recuperation from the donor [94, 95]. This improvement link with the TLM preservation could explain the hypoxia reduction, which increased the anti-apoptotic gene expression and inhibited pro-apoptotic gene expression [96]. However, since 2007 the results of such methods has been controversy and several islet isolation laboratory withdrawn this theory.

#### 1.4.3.2.2.4 Digestion

The enzymatic dissociation of acinar tissue by collagenase is a substantial step in the isolation of pancreatic islets. Although essential collagenase components have been purified, the variability in the activity of different batches limits long-term reproducibility of isolation success. Deterioration of enzyme activity with storage means that most islet isolation teams spend a significant amount of time and resources in batch-testing enzymes to find the occasional enzyme batch that is optimal. In islet transplantation, it is essential that for any pancreas, the collagenase digestion phase releases large numbers of viable islets that are completely free of exocrine tissue and that are not fragmented [97].

Islet yields were further improved by the development of the automated method. This method uses a combination of collagenase digestion and mechanical dissociation and has become the “gold standard” for human islet isolation. Jingu *et al.* have reported higher islet yields from the canine pancreas when the collagenase distended pancreas is mechanically chopped into small fragments immediately before being placed into the automated digestion system. This combined method significantly reduces the digestion time, thereby reducing islet fragmentation induced by digestion of the collagen contained in islets [98].

#### 1.4.3.2.2.5 Purification

The purification process is one of the most difficult procedures in islet isolation. It has been shown that half of the islets isolated from the pancreas are loss during this step [99]. In addition, islets are exposed to mechanical, enzymatic, osmotic and ischemic stresses during the purification procedure that cause cellular damages, functional impairment and eventually lead to an overall reduction of the viable islet mass that engrafts [100].

Currently, a continuous density gradient purification method is used with top loading using COBE 2991 cell. The original cell separator COBE 2991 was not refrigerated, and islets were exposed to inappropriately high temperatures during the purification step of the isolation process. Later, a cooling system was added to the COBE 2991 and significantly increased the viability and insulin secretion in response to glucose. Low temperature protects the cells from eventual toxicity of Ficoll and UW solution. A recent modification of the separation technique involves creating the density gradients from two solutions which are made up from combinations of Ficoll (1.100 g/ml) and UW solution improved the yield. Although, this



modification reduces the density range over which the gradient is created, it allows more digest material to be loaded and an effective separation in one purification run [101].

#### 1.4.3.2.2.6 Culture

Islets are cultured for maximum 48h before transplantation at the density of 150 IEQ/cm<sup>2</sup> and under 2mm of medium maximum to promote oxygen exchanges.

It has been described that this tissue has a very short lifespan in culture, because it is highly sensitive to lack in oxygen and nutrients. Islets cultured in standard conditions are exposed to sharp oxygen partial pressure gradients that may range from surface normoxia (142 mmHg) to central anoxia (0 mmHg).

#### 1.4.3.2.2.7 Islet infusion

The most utilized method of islet infusion is percutaneous transhepatic intraportal injection. It is performed by interventional radiography or laparotomy in the portal vein and 10,000 islets equivalents (IEQ) are required per kilogram patient body weight. To reach this number, several infusions are needed (two or three from successive donors). Some sites, including Lille (France) have switched from percutaneous puncture to microsurgery via intragastric route to reduce complications such as gallbladder puncture and haemorrhage [102]. It has been shown that the more islets are infused during the first transplantation the longer graft survive, therefore the threshold for the first infusion is 5,000 IEQ/kg body weight [103]. After completing the pancreatic islet infusion in the portal vein, islets are embolized and as a consequence there is a tissue rearrangement over time and re-vascularization of islets which permits their function. The liver is the site of choice for islets transplantation, although muscle was tried in autotransplantation as well [104]. Those sites are well vascularized which is important for the graft survival. However, in the liver, there is a tremendous loss of islets post-infusion due to IBMIR and vascularization time in short term [105], and due to function of the liver detoxification center and recurrence autoimmunity [106].

### 1.4.3.2.3 Islet transplantation and graft loss

#### 1.4.3.2.3.1 Rejection

The principal foreign agent recognized by the recipient is the foreign human leukocyte antigen (HLA). These antigens are presented in the cells and are unique in each person. It is uncommon two persons with identical HLA. In organ transplantation, the differences in HLA between donor and recipient stimulate the activation of immunologic rejection. In order to avoid this rejection, it is recommended to perform a donor specific cross match and a panel of reactive antibody (PRA) test on the serum prior to transplantation.

There are different types of rejection in function of the immunologic mechanisms, type of damage in the graft and time when the rejection occurs [107]:

- Acute rejection: it manifests around one week after transplantation. T cells from the host are already differentiated and antibodies are produced after one week. These T cells lyse the graft cells and produce cytokines that recruit other inflammatory cells, causing necrosis or allograft tissue. Depending of the earliness of the treatment, the damaged can be reverse or not.
- Chronic rejection: is the principal type of rejection. It occurs around months to years after transplantation. It is produced by the fibrosis of the transplanted tissue's blood

vessels. Various mechanisms have been proposed to account for the chronic rejection process. These include the wound healing process, delayed type hypersensitivity reaction with activation of T helper cells and B cells leading to activation of macrophages and the secretion of tissue growth factors, antibody-mediated humoral immunity, and endothelial cell damage leading to ischemia. Most recipients must take immunosuppressive drugs for the rest of their lives, and even that may not be enough to combat chronic rejection.

#### 1.4.3.2.3.2 Vascularization

As stated before, islets are highly vascularized. This vasculature gets disrupted during the process of islet isolation and culture, which causes an accumulation of endothelial fragments and compromises perfusion in the core of islets. Therefore, rapid vascularization is crucial for islet engraftment, survival, and function post-transplantation. Successful islet graft has been observed to regenerate the microvasculature within 10 to 14 days of transplantation. During the initial few days of transplantation, islet oxygenation depends only on the diffusion of oxygen and nutrients from the periphery. This limitation leads to lower oxygen and nutrient supply in the inner core of islets and ultimately leads to hypoxia and cell death.

Islets promote their own revascularization by secreting proangiogenesis mediators such as vascular endothelial growth factor (VEGF) and its receptors. Revascularization of islets post-transplantation occurs from the surrounding host tissue vasculature [108].

#### 1.4.3.2.3.3 Immunosuppressive regimen

Immunosuppressive treatment is needed to prevent rejection of the transplanted pancreatic islets. This treatment starts before islet transplantation with the administration of anti-IL2 receptor antibody (Daclizumab) during several months with the mix of low doses of Rapamycine (sirolimus), which inhibits the IL-2 response and FK506 (Tacrolimus), a calcineurin inhibitor, blocking the IL-2 production.

The loss of islet functionality and the islet vascularization delay are induced by immunosuppressive treatments which are deleterious for the medium-long term survival of the transplanted islets. In fact, Tacrolimus is able to inhibit the insulin mRNA transcription in the rat islets *in situ* [109] and decrease the functionality of human islets transplanted in mouse [110]. Rapamycine is used at a concentration of 10 ng/ml in order to avoid islet rejection, and it is able to decrease proliferation in  $\beta$  cells transplanted in rats [111]. Other studies have shown that Rapamycin can have anti-angiogenic capacities [112]. All these results suggest that the progressive loss of function in islets could be due to the immunosuppressive treatment. Also, the effects from tacrolimus and sirolimus are deleterious for islets survival just after transplantation where islets are stressed after isolation and are in a recovery phase [113].

#### 1.4.3.2.3.4 IBMIR

Currently, the liver is the choice for islet transplantation. However, there is a high islet loss due to IBMIR. IBMIR as a result of the introduction of islets into the portal circulation induced cell death, which involves a thrombotic and inflammatory reaction characterized by binding of platelets to the islet surface and infiltration of leukocytes in the islets [114]. Strategies to reduce IBMIR, a process triggered by tissue factor expression on the surface of islets, include the use of nicotinamide, low molecular weight dextran sulfate thrombin

inhibitor and heparin coating of islets. However, IBMIR remains a critical barrier to the function of  $\beta$  cells after transplantation into a vascular site, particularly as activation of the innate immune response is a known trigger of the equally problematic adaptive immune response [115].

In order to avoid those limitations, encapsulation has been developed for the last 20 years.

## **1.5 Islets encapsulation: Bioartificial pancreas**

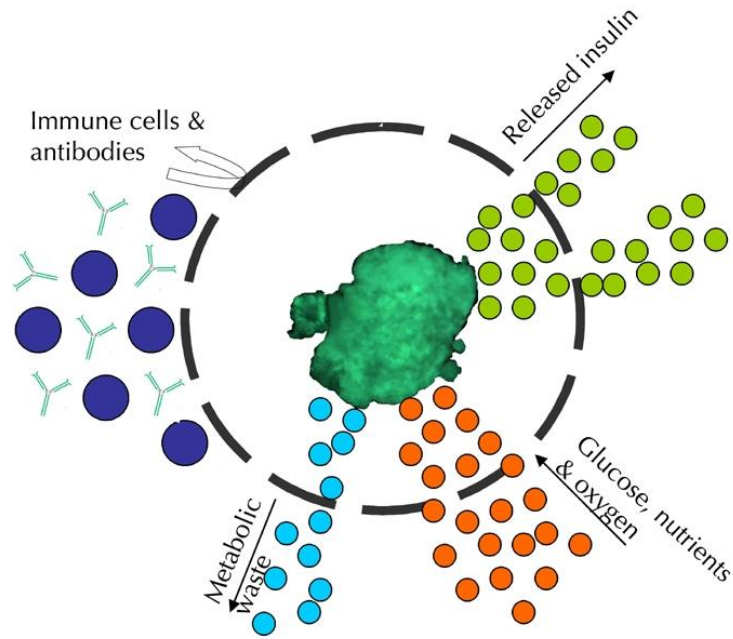
### **1.5.1 Concept**

In 1930, Bisceglie *et al.* demonstrated xenotransplantation was possible using polymer structure. Then, mouse cells could survive in a guinea pig without rejection [116]. From this time, the concept of immunoisolation has been proposed and different types of encapsulation devices have been developed.

Islet immunoisolation for transplantation is a strategy for type 1 diabetes to prevent the tissue destruction by the recipient immune system, blocking the access to cells such as T cells and cytokines with the use of encapsulation technology. Immunoisolation enables also the use of alternative source of cells producing insulin (xenogeneic islets, stem cells or  $\beta$  cells generated *in vitro*) for transplantation, to avoid the problem of lack of donors and the loss of islets during isolation.

The challenge of such encapsulation is to permit crossing of molecules such as insulin, glucose and gases while prohibiting the passage of immune messengers. In this way, the immune system cannot recognize the non-self islets preventing the allograft rejection mediated by T cells [117]. Encapsulation device is made of biocompatible and semi permeable materials which permit the bidirectional flow through the device, in order to be functional and being able of removing waste (**Fig. 1-32**).

In encapsulation technology the most extensively studies has been performed on isolated pancreatic islets for the treatment of type 1 diabetes mellitus.

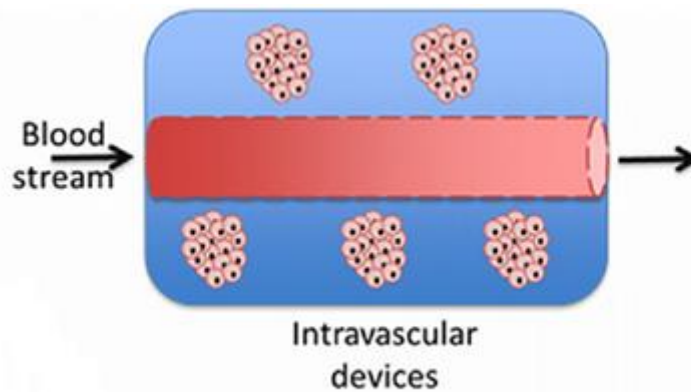


**Figure 1-32 Encapsulation principle [117]**

Bioartificial pancreas has been proposed for type 1 diabetic patients in order to control automatically their blood glucose, like a healthy pancreas. The purpose of this medical device is the same than islet transplantation, *ie*: to normalize the blood glucose in patients with inefficient insulin therapy [118]. The design of the device could be intravascular or extravascular.

### 1.5.2 Intravascular device

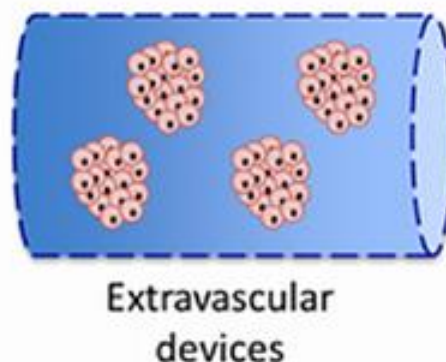
In intravascular devices, islets are encapsulated in a semi-permeable membrane with many artificial capillaries made of polyacrylonitrile and polyvinylchloride copolymer. The device is connected to the host systemic circulation by vascular anastomoses creating an intravascular shunt. One of the major advantages of the devices is the proximity to the recipient's blood stream that provide islets nutrients and oxygen and a quick response to high levels of glucose in the blood. The major obstacle is the intervention in the blood vessels, the device can disturb the blood stream and as a consequence activation of the coagulation factors and decreases the membrane permeability, which can produce thrombus formation and produce islet necrosis, requiring anticoagulation therapy (**Fig. 1-33**) [119].



**Figure 1-33 Intravascular devices [120]**

### 1.5.3 Extravascular device

In this kind of device, islets are inside a diffusion chamber and the creation of the intravascular shunts is not needed. The surgical risk is minimum; the retrieval is easier than intravascular device. Device can be implanted in different sites and there is no risk of thrombosis. However, disadvantages such as decrease in islet function due to fibrosis, lack of immediate vascularization, and extended nutrient/hormone diffusion time characteristic of chamber design need to be addressed [121]. Research is concentrated on optimizing chamber designs [122], improving vascularization or implanting the device prior to islet insertion [123] or improving oxygen transfer [124]. Based on the size of the device, they can be classified in microcapsular devices or macrocapsular devices (**Fig. 1-34**) [117].

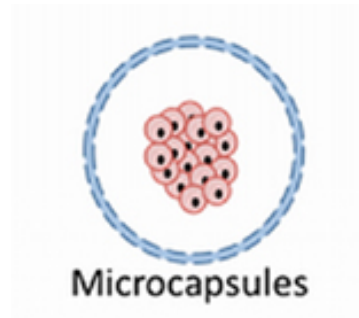


**Figure 1-34 Extravascular devices [120]**

#### 1.5.3.1 Microencapsulation

Microcapsules are spherical and generally one or few islets are encapsulated in a semi-permeable membrane. The simplicity in the manufacturing process permits to change the pore size, permeability and thickness. There are no surgical risks, they are injected directly in the peritoneal cavity or implanted with minimal invasive surgery. However, the major disadvantage is the difficulty to retrieve the microcapsule from the recipient if there is pericapsular fibrotic overgrowth, biocompatibility, efficiency and microcapsule resistance. Microcapsule membranes avoid all the inter-insular communications that could be deleterious

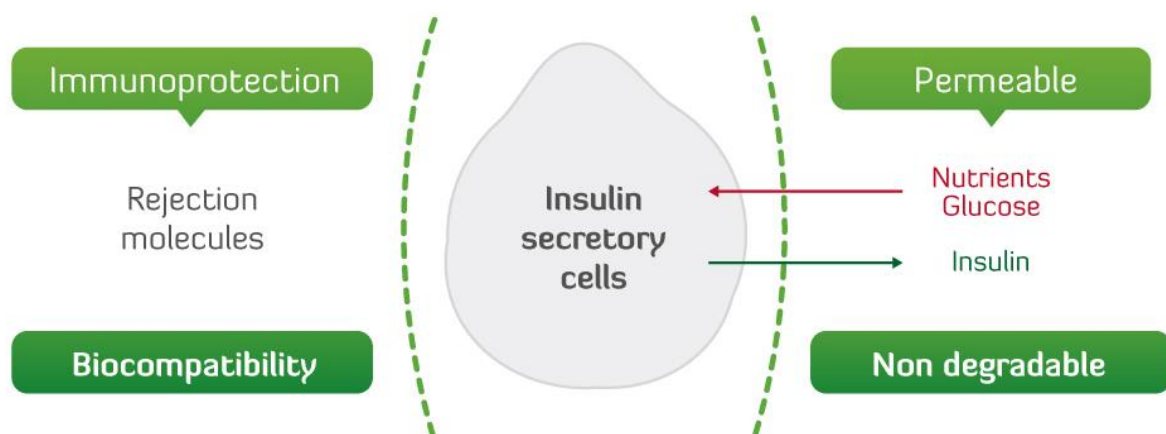
for islets. Islets microcapsules are made of biomaterials like alginate [125], agarose, chitosan, methacrylic acid, methyl methacrylate and polyethylene glycol and 2-hydroxyethyl methacrylate (HEMA) [126]. Transplantation of microencapsulated islets is generally done in the peritoneal cavity of animals (mice, rats, monkeys and dogs) (**Fig. 1-35**) [127].



**Figure 1-35 Microencapsulation [120]**

### 1.5.3.2 Macroencapsulation

Macrovascular devices have different shape but the principle is to gather islet or cells in one or few devices, and not for an individual protection. The module and the membrane have to be biocompatible, and the porosity of the membrane must allow the entry of glucose and oxygen and the exit of insulin. The exchange has to be rapid in order to respond to an increase of glucose in the blood. The size pore has to be limited to restrict the entry to cells implicated in the rejection reaction. One of the major advantages of extravascular macrocapsules is their easy retrievability after implantation [117] (**Fig. 1-36**).



**Figure 1-36 Bioartificial pancreas concept**  
Source: Defymed

### 1.5.4 Biocompatibility

One of the reasons of encapsulated islet death is the fibrotic overgrowth if the capsule is not biocompatible. After transplantation, macrophages and fibroblasts are responsible of the fibrosis and around 40% of the transplanted islet mass is lost. The lack of biocompatibility could be due to the purity or shape of the encapsulated material.

### 1.5.5 Immunoprotection

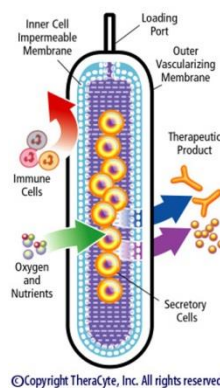
Encapsulated islets should be protected from rejection mechanisms and autoimmune destruction. Even if the semi-permeable membrane should protect islets against immune cells and antibodies, islets are still vulnerable to small molecules like chemokines/cytokines or nitric oxide (NO) that can cross the membrane and go into the device. These molecules are also produced by islets themselves, which can cross the membrane, go outside the device and attract macrophages. This process is called chemotaxis and can produce graft failure. Those chemoattractants like alpha 1,3-galactose and MCP-1 activates macrophages, which release pro-inflammatory cytokines, interleukin-1 (IL-1 $\beta$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), interferon gamma (IFN- $\gamma$ ), and NO [128, 129]. In order to prevent the cross of these small molecules the pore size of the membrane should be reduced but, if the pore size is too small the diffusion of nutrients and insulin can be affected. Thereby achieving the right pore size in the membrane is crucial for islet survival [117].

Different strategies to improve immunoprotection have been proposed, like co-encapsulation islets with erythrocytes and sertoli cells which release immunosuppressive factors [130] and also secretion of anti-inflammatory molecules by genetic engineering islets reduced  $\beta$  cell damage from molecules secreted by macrophages [131].

### 1.5.6 Prototypes of bioartificial pancreas

#### 1.5.6.1 TheraCyte<sup>®</sup>

Tubular macrocapsular device with cylindrical shape and smooth surface prevent fibrosis overgrowth and enhance the compatibility. It is fabricated from biocompatible membranes, which protect allogeneic cells from rejection by the recipient. It has a width of 3 cm, length of 8 cm and a volume of 250  $\mu$ L, which gives the height 0.0104 cm. One of these devices will not be enough to carry a sufficient amount of IEQ, since it is stated that their 250  $\mu$ L device is able to carry 31,250 human IEQ. Therefore, large numbers of devices are needed to reach normoglycemia due to the low islet seeding density [132] (**Fig. 1-37**).



**Figure 1-37 TheraCyte<sup>®</sup>**  
Source: <http://www.theracyte.com>

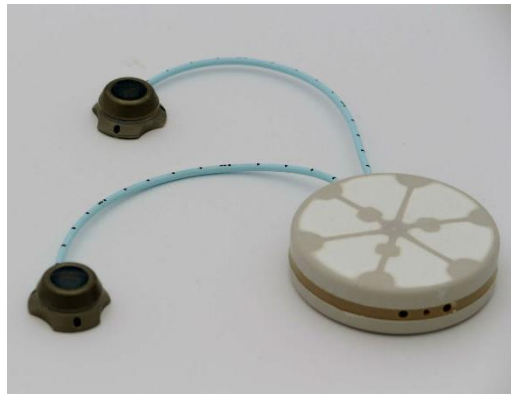
Pig islets from the fetal, neonatal and adult pancreas are the commonly source of islets for xenotransplantation in diabetic animal models. Xenotransplantation studies using TheraCyte<sup>®</sup>

have been demonstrated the survival of porcine islets in cynomolgus monkeys for up to 8 weeks [133]. Recent studies with TheraCyte<sup>®</sup> showed human fetal pancreatic tissue transplanted in rodents resulted in their differentiation into insulin-producing cells. The major disadvantages for the xenotransplantation is the xenozoonosis, the risk of developing new diseases transmitted from animal to human. Patients and monkeys transplanted with encapsulated porcine tissue showed no evidence of porcine endogenous retroviruses (PERV) infection [134].

Other donor sources of islets could be stem cells. In fact, there are different studies under development using adult stem cells or embryonic stem cells (ES) for cell therapy. Viacyte<sup>®</sup> produced hES-derived pancreatic progenitors which generated endocrine cells, those cells ( $5 \times 10^6$ – $2 \times 10^7$ ) were tested in the device TheraCyte<sup>®</sup> implanted in diabetic mice and after 2 months there was a normalization of blood glucose levels [135].

#### 1.5.6.2 Beta-air

It has the shape of a disc with two compartments containing islets and an oxygen central module which supplies oxygen through an exterior pump which is a disadvantage for the patient. The device had the external dimensions of 68 mm and 18 mm for diameter and height respectively. The islets modules are 0.060 cm thick with a diameter of 4.9 cm (capacity of 75,000 IEQ/module, 4.000 IEQ/cm<sup>2</sup>), which gives a volume of 2.26 mL (1.13 mL per islet module) [136] (**Fig. 1-38**).



**Figure 1-38  $\beta$ -air II**  
Source: [www.-o2.com](http://www.-o2.com)

Experiments performed with the  $\beta$ -air II device filled with islets showed normoglycaemia levels in diabetic rats for period up to 6 months, after device retrieval, there were no signs of inflammation and showed visual evidence of vasculature at the site of implantation [137].

#### 1.5.6.3 MAILPAN<sup>®</sup>

Defymed owns the IP (intellectual property) and exploitation rights related to the MAILPAN<sup>®</sup> bioartificial pancreas. Several patents describing MAILPAN<sup>®</sup>'s components, surface coating or design are under filing and/or international extension by Defymed. Therefore, for confidentiality reasons and in order to keep safe the exploitation rights of the MAILPAN<sup>®</sup> device by Defymed, CEED is not allowed to communicate any details related to the MAILPAN<sup>®</sup>'s components, surface coating or design. CEED and Defymed are bound by a consortium agreement via the European project, BIOSID signed on June 15, 2012.



This is a circular device with an inlet and an outlet chamber which permits the refilling of the device without major surgery. The size is around 15 cm in diameter for a thickness of few millimeters. The device is made of selective membranes (**Fig. 1-39**).



**Figure 1-39 MAILPAN®**  
Source: [www.defymed.com](http://www.defymed.com)

The characteristics of the actual MAILPAN® membrane have been made testing different properties from different membranes. The first membrane tested was the AN69 in a macrovascular device with an appropriate geometry allowing it to test various flat artificial membranes under satisfactory conditions. The AN69 has a molecular weight cutoff of the membrane evaluated at 65,000 daltons and a pore size of 10-14 Å. This membrane was supposed to be permeable to insulin (5,800 daltons) and glucose (180 daltons) while preventing the passage of immune cells (10-20 µm in diameter) and antibodies (180,000 for immunoglobulin G) [138]. Its thickness (20 µm) allowed both a rapid diffusion and an important hydraulic permeability (40 mL/h/min/mmHg) [139].

Studies performed showed that implantation of the AN69 membrane in the peritoneal cavity in rats reduced its permeability towards glucose and insulin by about 50% [140]. Cytological studies after device retrieval showed an important cellular colonization of the external surface of the membrane. 80% of the cells adhering were macrophages and the rest fibroblasts [139].

After these results, modifications of the physicochemical properties of the AN69 membrane induced by the Corona discharge treatment were performed. This treatment is based on radical and ionic interactions between atmosphere and surface and it is performed in air. The exposure time is shorter enough to preserve the structural integrity of the membrane. The surface treatment using Corona discharges did not modify the glucose permeability of the AN69 membrane. However, improvement in insulin diffusion was found. Biocompatibility test after the treatment were performed and 1 year after implantation the structure of the treated membrane was preserved without alteration of the fiber network or cellular adhesion at the surface of the membrane. This treatment seemed to decrease the number of glycerol molecules at the membrane surface without alteration of the initial AN69 membrane biocompatibility [141].

The efficiency of the AN69 membrane as a barrier to retain chemoattractants was evaluated by comparing in the supernatants the chemotactic effect of free and encapsulated islets in AN69 membrane. Lower chemotactic response of the encapsulated islets indicated that

diffusion of soluble factors release by pancreatic islets was only partially prevented by the AN69 membrane as a consequence of the pore size, which allows the passage of proteins secreted by islets or cytoplasmic proteins released by dead cells. However, culture empty devices induced no release of chemotactic molecules able to stimulate peritoneal macrophages. These results confirmed that the membrane AN69 was biocompatible and inert and the attraction of macrophages was induced by pancreatic islet [142, 143].

Xenotransplantation of 1,000 rat islet in diabetic male mice were performed using the AN69 membrane. The fasting glycemia level of the 8 diabetic mice transplanted was normalized 24 hours after transplantation of encapsulated islets. Normal fasting glycemia level was observed in 50% of the mice on day 19 and 30 [138].

Other kind of membrane was tested because the hydrophilic AN69 membrane must be kept permanently in water to avoid structure modification after exposure to air. Thus, other treatments under vacuum were prohibited rendering its handling very difficult. Therefore, a polycarbonate membrane was selected to improve the permeability. A plasma treatment was applied to the polycarbonate membrane which increased the hydrophilic properties and improved the glucose diffusion through the membrane without modification of the material structure and biocompatibility was preserved. Copolymer adsorption treatment at the polycarbonate membrane surface was also performed to improve hydrophilic properties. This treatment improved glucose and insulin diffusion but was not biocompatible because of its instability after *in vivo* implantation. However, the combined surface treatments, plasma and copolymer adsorption, conferred a high stability to the polycarbonate surface insuring the preservation of the biocompatibility [144].

## **1.5.7 Issues related to macroencapsulation**

### **1.5.7.1 Transplantation site**

Commonly, transplantation of encapsulated islets is performed in the intraperitoneal cavity; the advantages of this site allow the transplantation with a laparoscopic. However, is not easy to find a site big enough to implant the macrovascular device and insure the contact with blood vessels. The problem of non-vascularized system in the intraperitoneal cavity becomes in a loss of graft functionality and a delay in insulin uptake into the blood stream.

Alternatives sites for transplantation of islets macroencapsulated have been studied, like the omental pouch to decrease diffusion barriers, subcutaneously and peritoneal cavity. Ludwig *et al.* transplanted macroencapsulated islets in the extraperitoneal cavity in humans; the islets graft survived and maintained glucose responsiveness during 10 months after transplantation [145]. The subperitoneal space is a large, unifying, anatomically continuous potential space that connects the peritoneal cavity with the retroperitoneum in the abdominal cavity. The subperitoneal space as a transplantation site is highly vascularized and metabolically active tissue that should provide a good exchange. Other investigators implanted the TheraCyte subcutaneously in rats and after 3 months the device was filled with rat islets. These results were compared with free islets implanted under the kidney capsule and they have showed that subcutaneous encapsulated islets were functional at least after 6 months after transplantation with no rejection compared with 5 days of survival in islets implanted in the kidney capsule which were rejected after 2 weeks [146].

### 1.5.7.2 Confinement

In order to reverse diabetes, a minimum in islet number is required, 15,000IEQ/kg when macroencapsulated [116]. However, density per cm<sup>2</sup> is critical since oxygen and nutrient availability depend on the number of islet. The more islets there are in a confined environment the more oxygen is consumed [147, 148]. In a device, the surface is limited by the clinical application, thus, devices need to be of reasonable size.

The number of islet is fixed by the quantity of insulin required to control glycemia and the size or the number of the devices is limited by the physiology. Different experiments have been already done with different islet concentrations using the bioartificial pancreas. Experiments performed with TheraCyte<sup>®</sup> device using 1,000 islets per device, was able to maintained normal blood sugar levels after 6 months in rats. However, the subcutaneous implantation of the device is not the best place to respond to changes in blood glucose levels [146]. To improve islet survival in a confined device, the  $\beta$ -air II device (filled with 2,300IEQ/cm<sup>2</sup>) was implanted in rats and it restored normal nonfasting blood glucose levels for a period of 58 days. This device has to be infused with oxygen once a day in order to prevent hypoxia [137].

### 1.5.7.3 Hypoxia

One of the major problems of the graft failure is the lack of oxygen. Despite the fact that islets represent only 1-2% of the pancreas, they receive 10-15% of the blood flow indicating that islets receive and consume high dose of the oxygen. The absence of revascularization and oxygen diffusion in encapsulated islets leads to chronic hypoxic stress. In this condition, insulin release and islet function is impaired; this explains why a larger number of encapsulated islets is needed than non-encapsulated islets to reverse diabetes.

Hypoxia pathway is activated during islets encapsulation, which is under the control of Hypoxia Inducible Factor-1 (HIF-1).

#### 1.5.7.3.1 Hypoxia Inducible Factor (HIF-1 $\alpha$ )

HIF-1 factor induced by hypoxia is a protein complex which enhanced the expression of specific genes sensible involved in the defenses against the lack of oxygen. Moreover, HIF-mediated pathways are implicated in cell growth and differentiation, survival and apoptosis, angiogenesis and glucose metabolism adaptation.

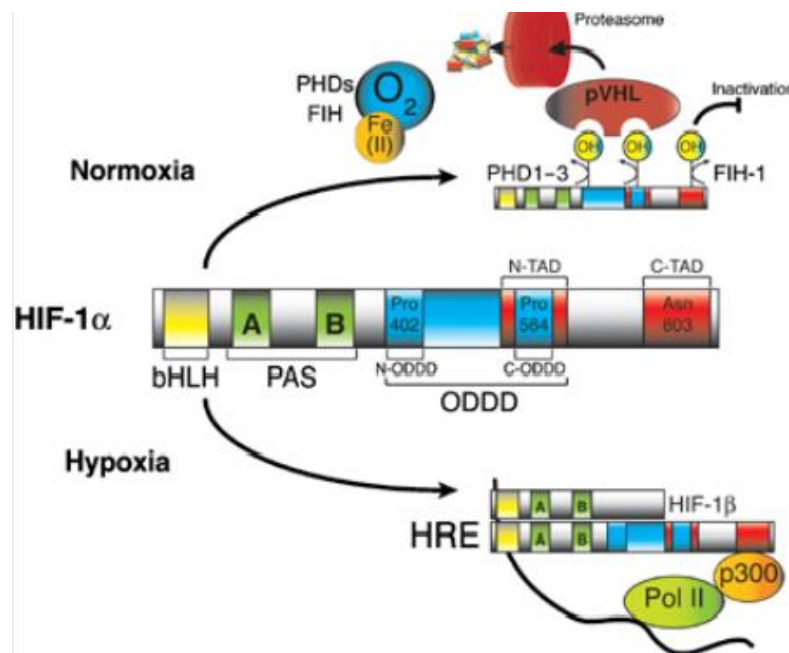
HIF is a heterodimer DNA-binding complex composed by two subunits: the constitutive HIF-1 $\beta$ , and the hypoxia inducible  $\alpha$  subunits, HIF-1 $\alpha$  or HIF-2 $\alpha$ . In hypoxia, the  $\alpha/\beta$  heterodimer binds to a core pentanucleotide sequence in the hypoxia response elements (HREs) of target genes. HIF-1 $\beta$  subunits are non-oxygen responsive nuclear proteins that also have other roles in transcription and HIF-1 $\alpha$  subunits are composed by important functional domains in order to regulate the gene transcription:

- Oxygen dependent degradation domain (ODD) responsible of the HIF-1 $\alpha$  degradation in normoxia by the proteasome [149]. A sequence of 15 amino acids important for HIF-1 $\alpha$  stabilization during hypoxia.
- A domain which allow the interaction with the PAS domain (N-terminal), leading the cytoplasmic retention.
- A sequence implicated in the translocation of HIF-1 $\alpha$  to the nucleus due to hypoxia.

Related to HIF-1 $\alpha$  expression, glycolysis is increased to produce more ATP and avoid energy deprivation in hypoxic cells. It has been reported that HIF-1 $\alpha$  is an important molecule to regulate  $\beta$  cell function in pancreatic islets even in normoxic conditions [150].

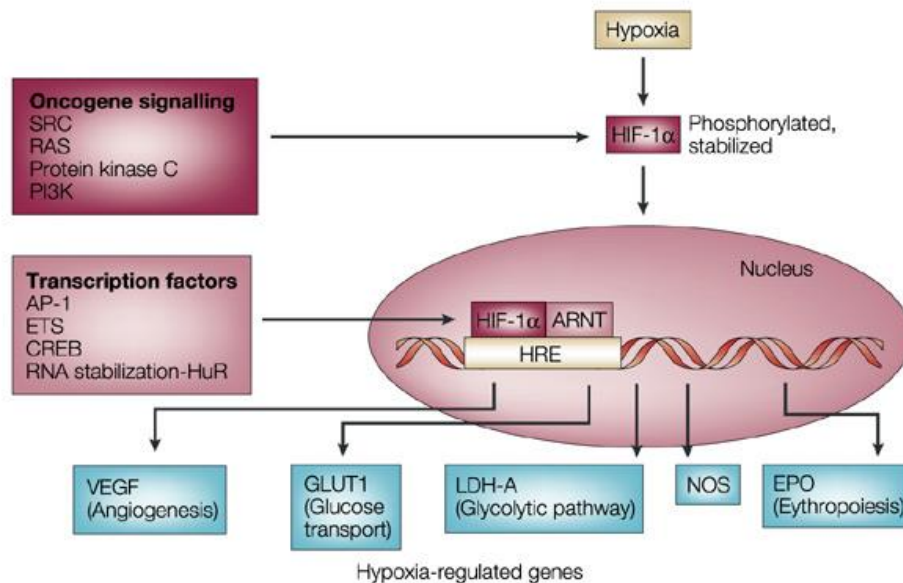
- Regulation of HIF:

Under normoxic conditions, HIF-1 $\alpha$  is continuously synthesized and degraded. There is a hydroxylation of two prolyl residues in the ODD of the  $\alpha$ -subunits. This oxygen dependent hydroxylation regulates the interaction with the von Hippel-Lindau tumor suppressor protein (pVHL). pVHL is the recognition of an E3 ubiquitin ligase complex that targets HIF-1 $\alpha$  for proteolysis by the ubiquitin proteasome pathway. Under low concentration of oxygen, the degradation of HIF-1 $\alpha$  is retarded. Prolyl hydroxylation (PHDs) is suppressed, HIF-1 $\alpha$  protein escapes proteasomal destruction from ubiquitination and can accumulate in the cytosolic. HIF-1 $\alpha$  is translocated to the nucleus and dimerizes with HIF-1 $\beta$ , which is not affected by hypoxia. The heterodimeric complex binds to the HRE in the promoter to enhance the sequences of target genes (**Fig. 1-40**) [151].



**Figure 1-40 Regulation of HIF-1 $\alpha$  protein by prolyl hydroxylation and proteasomal degradation [151]**

Sometimes simple diffusion of oxygen is inadequate for metabolic demands and the first defense set up by hypoxic cells consists in triggering the growth of vessels. HIF is a VEGF transcription factor and plays a major role in VEGF production when is needed to promote angiogenesis, one of the major processes involved in new blood vessels (**Fig. 1-41**) [152].



**Figure 1-41 Factors involved in HIF-1 activation of hypoxia-response genes [153]**

### 1.5.7.3.2 Angiogenesis

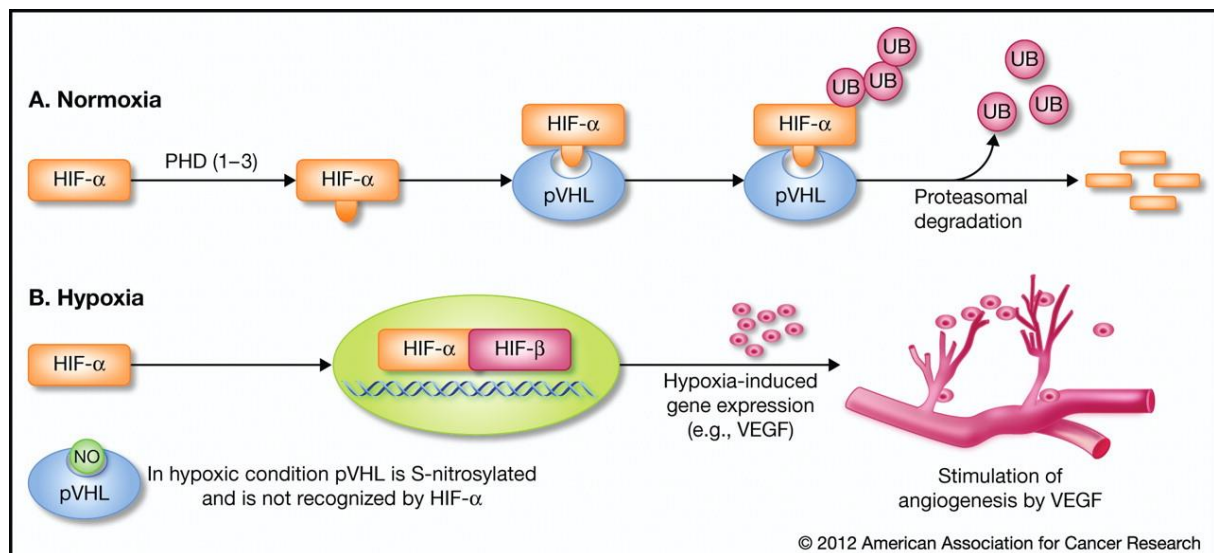
Angiogenesis is important for vascularization of the device. It is a complex process that includes remodeling of the ECM, migration and proliferation of endothelial cells, capillary tube formation and maturation of blood vessels. This process is initiated by angiogenic factors like VEGF or VEGF-A and related molecules (VEGF-C and VEGF-D).

After vascular lesion, ischemia or tumor cells multiplication, cytokines and growth factors are released in the medium and activated the endothelial cells. Those cells guide the developing capillary sprout through the ECM toward an angiogenic stimulus such as VEGF-A. Long, thin cellular processes on tip cells called filopodia secrete large amounts of proteolytic enzymes, which digest a pathway through the ECM for the developing sprout. The filopodia of tip cells are heavily endowed with VEGF-A receptors, allowing them to “sense” differences in VEGF-A concentrations and causing them to align with the VEGF-A gradient. When a sufficient number of filopodia on a given tip cell have anchored to the substratum, contraction of actin filaments within the filopodia literally pull the tip cell along toward the VEGF-A stimulus. Meanwhile, endothelial stalk cells proliferate as they follow behind a tip cell causing the capillary sprout to elongate. Vacuoles develop and coalesce, forming a lumen within a series of stalk cells. These stalk cells become the trunk of the newly formed capillary. When the tip cells of two or more capillary sprouts converge at the source of VEGF-A secretion, the tip cells fuse together creating a continuous lumen through which oxygenated blood can flow. When the local tissues receive adequate amounts of oxygen, VEGF-A levels return to near normal. Maturation and stabilization of the capillary requires recruitment of pericytes and deposition of ECM along with shear stress and other mechanical signals [154].

In humans, there are four VEGF-A isoforms consisting of 121, 165, 189 or 206 amino acids which are generated by alternative splicing of a single gene. They are characterized by the link capacity to heparin and other proteoglycans which are components of the ECM. The native VEGF is a heparin-binding homodimeric glycoprotein of 45kDa. VEGF 165, the most abundant, lacks the residues encoded by exons 6 and 7, it has intermediary properties and it is

secreted by the ECM. VEGF 189 is found attached to the ECM while VEGF 121 and 165 are soluble. The others family members VEGF-C and VEGF-D, have an important role in the angiogenesis tumoral. VEGF-C is stimulated by growth factors like PDGF and EGF but not for hypoxia [155].

In islet encapsulation, there is an inadequate vascularization of the device the first days after implantation, which results in the dysfunction and death of the islet tissue. Most of the revascularization takes place over a period of 30 days with almost all the vessels arising from the recipient. Bioartificial pancreas as MAILPAN<sup>®</sup> and Viacyte<sup>®</sup> implant the device empty and 30 days after, the islet infusion is performed in order to have a good vascularization. The formation of new blood vessels optimizes the provision of nutrients and oxygen to the devices and provides access for systemic delivery of insulin. It has been studied that islets exposed to hypoxia have an increase in VEGF levels during the first few days of encapsulation as a stimulus to initiate the growth of blood vessels into the graft and the development of fenestrations. However, the presence of VEGF in normal islets suggests it may be important for maintenance of normal islet vasculature (**Fig. 1-42**) [156].



**Figure 1-42 VEGF activation induced by hypoxia**  
**Source: American Association for Cancer Research, 2012**

#### 1.5.7.4 Strategies to improve hypoxia in islet macroencapsulation

##### 1.5.7.4.1 Pro-angiogenic factors

Overexpression of VEGF by cellular therapy, local addition or genetic therapy could be a good strategy to increase oxygenation. VEGF should be able to cross the semi-permeable membrane of the device and promote devices vascularization. Pancreatic islets co-encapsulated with exogenous VEGF using a macroencapsulated device with the AN69 membrane minimize the hypoxic stress and prevent apoptosis. This study showed the decrease in hyperglycemia in mouse during 28 days compared to islets without VEGF [157].

#### 1.5.7.4.2 Hypoxia resistant cells

Experimental approaches to overcome this problem include the use of hypoxia-resistant islet cells. Pancreatic islets from Tilapia, a tropical teleost fish, tolerate lower oxygen tension than mammalian islets. Fish islets cultured in deoxygenated medium were viable after 72 hours compared to rat islets which underwent near total necrosis and fragmentation after 24 hours culture. Fish islets cultured under hypoxic conditions ( $PO_2 = 27.8$  mmHg) and then transplanted into diabetic mice were viable 25-day follow-up period [158].

#### 1.5.7.4.3 Oxygen delivery

Co-encapsulation of photosynthetic oxygen generator algae with islets resulted in greater insulin response to glucose [159]. In another studies, investigators proposed the effect of a combination of growth-hormone-releasing-hormone agonist and a controlled oxygen supply on the function of a bioartificial chamber. This study showed the normalization of blood glucose in diabetic rodents for up to 3 months after subcutaneous transplantation [160].

The use of islets engineered to contain an intracellular oxygen carrier such as myoglobin can be also a good strategy to improve oxygenation. Tilakaratne *et al.* showed, using a mathematical analysis, that myoglobin facilitated oxygen transport has the potential of increasing the oxygen concentration at the center of an islet with an effective radius of 100 microm by 50%. Also including myoglobin in the alginate gel would beneficially improve the flux of oxygen to the encapsulated cells [161].

Inclusion of PFCs or hemoglobin in islet encapsulation is a useful strategy for overcoming oxygen limitations and ensuring cell viability and functionality both for large devices (>1 mm) and over extended time periods. These oxygen carriers could be useful for improving and stabilizing oxygen supply in a wide range of cell types and devices.

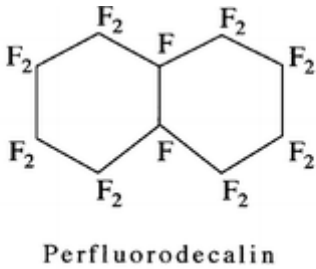
##### 1.5.7.4.3.1 Perfluorocarbons (PFCs)

Rat islets cultured during 48 hours with PFCs showed an improvement of viability and a higher stimulation index compared with islets cultured without PFCs. After transplantation 50% of the recipients became normoglycemic compared with the 14,3% of control recipients. As well, in order to provide oxygen to the site transplantation to improve the graft survival, PFCs saturated with oxygen were injected in the transplantation site before to transplant islets. PFCs emulsions have been used also to prevent hypoxia of pancreatic cells. Rat islets were incubated for 3 days in presence of PFC emulsion and islet viability and functionality was preserved for several days after isolation [162].

To enhance oxygen permeability of the encapsulating material, PFC oxygen vectors, specifically perfluorooctyl bromide (PFOB) immobilized in an alginate matrix was used in cell encapsulation. Oxygen uptake rates of HepG2 cells were enhanced with 10% PFOB addition under both 20% and 5%  $O_2$  boundary conditions compared to alginate matrix alone [163].

Perfluorodecalin (PFD) ( $C_{10}F_{18}$ ) is a polycyclic perfluoroalkane. It is a derivative of decalin on which all of the hydrogen atoms are replaced by fluorine atoms. Its density (1.92 g/cm<sup>3</sup>) and viscosity (2.61 cS) [164] are higher than water. This molecule is very stable, biologically inert and is easily eliminated by the body. PFD have been found to dissolve high

concentrations of oxygen that is released when the partial pressure of oxygen is reduced in the surrounding environment (Fig. 1-43).

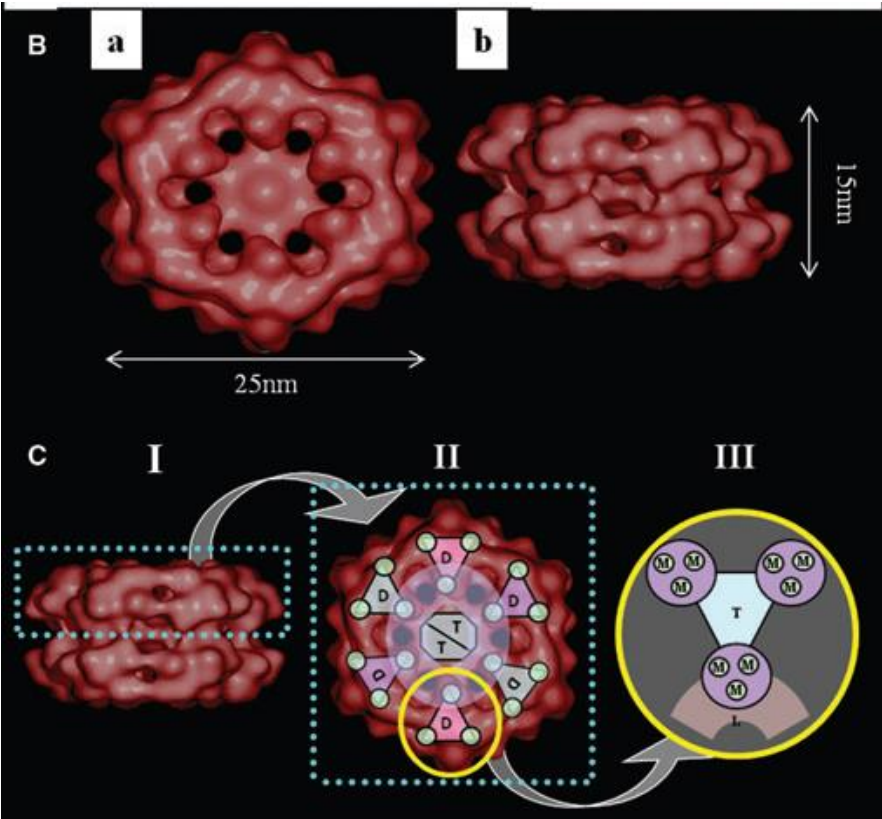


**Figure 1-43** Chemical structure of cyclic perfluorocarbon, perfluorodecalin (PFD) [165]

1.5.7.4.3.2 HEMOXcell<sup>®</sup>

HEMOXcell<sup>®</sup> is a hemoglobin found in the marine invertebrate blood *Arenicola marina*. This hemoglobin is a high molecular weight protein (~3,600 kDa), composed of 156 globins and 44 non-globin linker chains carrying up to 156 O<sub>2</sub> molecules when saturated. HEMOXcell<sup>®</sup> release O<sub>2</sub> according to a simple gradient, providing the environment the right amount of O<sub>2</sub> [166].

HEMOXcell<sup>®</sup> has been tested in mice bearing human-derived subcutaneous tumors where oxygen levels were lower. Intravenous injection of the hemoglobin showed an oxygenation increased in tumor tissue. This effect was 15 min post-injection demonstrating the ability of HEMOXcell<sup>®</sup> to diffuse within poorly vascularized tissues and to behave as a potent oxygen carrier toward vertebrate tissues, without inducing any obvious side-effects (Fig. 1-44) [166].



**Figure 1-44** Structure and conformation of *Arenicola marina* hemoglobin [167]



The benefit of this oxygen carrier has been tested also in organ preservation. HEMOXcell<sup>®</sup> supplementation on cold stored cells *in vitro* and on kidney grafts stored with either UW or HTK solution increased cell resistance to ischemia reperfusion injury and it is correlated with improved early function recovery and better long-term outcome with less chronic fibrosis and amelioration of function. HEMOXcell<sup>®</sup> addition increased cellular ATP content, maintaining the energetic metabolism and decreasing the need to switch from mitochondrial respiration to anaerobic glycolysis and protecting the mitochondria [167]. Low dose of HEMOXcell<sup>®</sup> significantly reduces serum creatinine levels, marker of kidney function, within the first 2 weeks and the intensity of ischemia-reperfusion injury improving the graft outcome [168].

However, this molecule has not been tested in islet preservation or encapsulation prior to transplantation.

#### 1.5.7.5 Inflammation

One of the major problems of the graft failure in encapsulation is islet inflammation. The substantial loss of  $\beta$  cell mass and function following encapsulation and transplantation constitutes a major impediment upon success of encapsulated islets as a treatment for type 1 diabetes. Main pathways related to islet inflammation are NF- $\kappa$ B and CREB pathway with different molecules implicated.

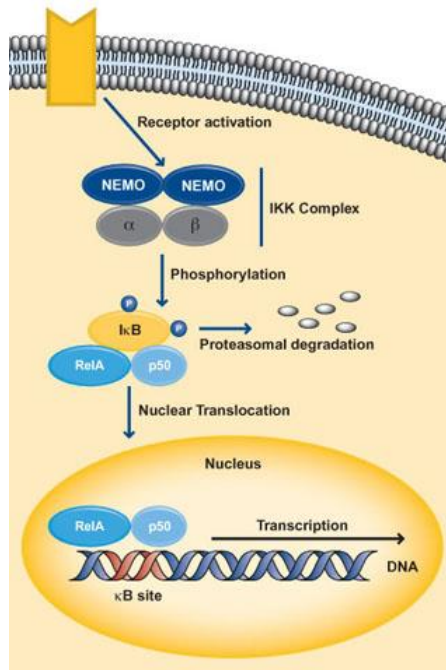
##### 1.5.7.5.1 The NF- $\kappa$ B signaling pathway

Nuclear transcription factor NF- $\kappa$ B is a transcription factor controlling expression of genes involved in inflammation, survival and death in a variety of cell types.

In mammals, the NF- $\kappa$ B family is composed of five related transcription factors: p50, p52, RelA (p65), c-Rel and RelB. These transcription factors share the Rel homology domain (RHD). The RHD is a platform where family members can form homodimers and heterodimers, enabling them to bind promoters and enhancer regions of genes to modulate their expression. RelA, c-Rel and RelB contain C-terminal transcriptional activation domains (TADs), which triggers gene expression. In contrast, p50 and p52 homodimers repress transcription unless they are bound to a protein containing a TAD, such as RelA, c-Rel or RelB or Bcl-3 (a related transcriptional co-activator). Unlike the other NF- $\kappa$ B family members, p50 and p52 are derived from larger precursors, p105 and p100, respectively [169].

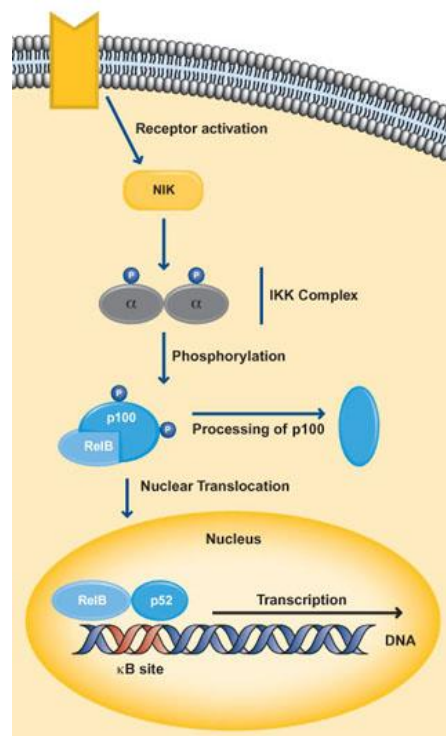
Two signaling pathways lead to the activation of NF- $\kappa$ B, known as the classical (canonical) pathway and the alternative (non-canonical) pathway:

- In the canonical signaling pathway, the binding of ligand to a cell surface receptor such as a member of the Toll-like receptor superfamily leads to the recruitment of adaptors (such as TRAF) to the cytoplasmic domain of the receptor. These adaptors in turn recruit the IKK complex which leads to phosphorylation and degradation of the I $\kappa$ B inhibitor. The canonical pathway activates NF- $\kappa$ B dimers comprising RelA, c-Rel, RelB and p50 (**Fig. 1-45**) [170].



**Figure 1-45 NF-κB canonical signaling pathway**  
Source: Abcam

- The alternative pathway is responsible for the activation of p100/RelB complexes and occurs during the development of lymphoid organs responsible for the generation of B and T lymphocytes. Only a small number of stimuli are known to activate NF-κB via this pathway and these factors include lymphotoxin B and B cell activating factor (BAFF) (**Fig. 1-46**) [171].



**Figure 1-46 NF-κB alternative signaling pathway**  
Source: Abcam

The common regulatory step in both of these cascades is activation of an I $\kappa$ B kinase (IKK) complex consisting of catalytic kinase subunits (IKK $\alpha$  and/or IKK $\beta$ ) and the regulatory non-enzymatic scaffold protein NEMO (NF- $\kappa$ B essential modulator also known as IKK $\gamma$ ). Activation of NF- $\kappa$ B dimers is due to IKK-mediated phosphorylation-induced proteasomal degradation of I $\kappa$ B, enabling the active NF- $\kappa$ B transcription factor subunits to translocate to the nucleus and induce target gene expression as cyclooxygenase 2 (COX-2), MCP-1 and Interleukin-6 (IL-6) [172].

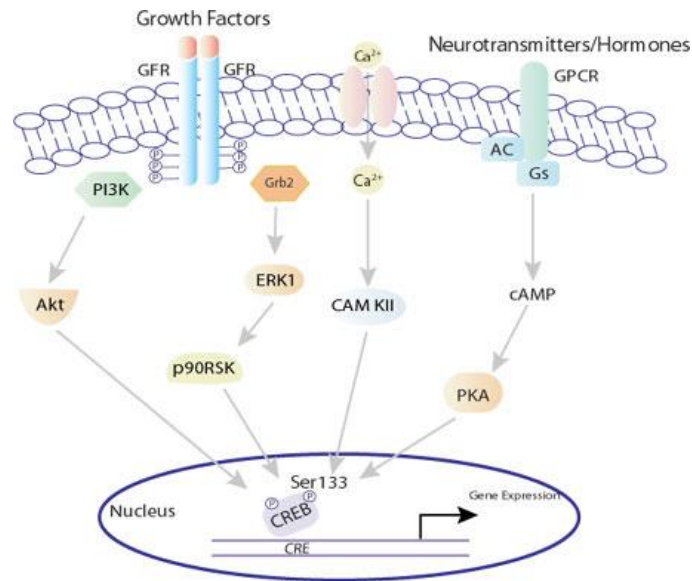
Pro-inflammatory status of islet post-isolation promotes cytokine secretion [173] which in return activates the NF- $\kappa$ B pathway. The regulation of NF- $\kappa$ B was showed to be involved in  $\beta$  cells dysfunction [174]. In addition, NF- $\kappa$ B has been shown to be involved also in islet survival and function during transplantation. One of the targets of NF- $\kappa$ B is iNOS, responsible for the production of NO, which triggers apoptosis in  $\beta$  cells. Moreover, NF- $\kappa$ B-dependent genes involved in glucose regulation are downregulated and genes involved in anti-graft immunity are up-regulated. Cyclooxygenase 2 is activated in NF- $\kappa$ B pathway with a negatively impact in glucose-stimulated insulin release and can induce islet apoptosis [175]. Hypoxia can also upregulate downstream genes in the NF- $\kappa$ B pathway, which are mainly related to  $\beta$  cell mass apoptosis [176].

#### 1.5.7.5.2 CREB pathway

Another transcription factor implicated in inflammation is CREB (cAMP-responsive element-binding protein) that regulates a wide variety of genes by binding to cyclic AMP response element (CRE) in the promoter region of genes, including COX-2, IL-6, the antiapoptotic gene bcl-2 and IRS-2.

Cytokines and chemokines implicated in chronic inflammation are known to induce apoptosis of pancreatic  $\beta$  cells. Studies demonstrated a decrease in bcl-2 promoter activity by cytokines in  $\beta$  cells suggesting a defect at the level of CREB resulted in activation of caspase-9 and exaggeration of cytokine-induced  $\beta$  cell apoptosis [177]. In addition, activation of hypoxia triggers hyperphosphorylation, ubiquitination and subsequently proteasome degradation of CREB, which mediates alterations in basal transcriptional activity [178].

Activation of CREB, is induced through phosphorylation mediated by kinases, such as PKA and ERK1/2. The phosphorylation of serine 133 is required for CREB-mediated transcription [179]. CREB has been reported to regulate the expression of the genes bcl-2 and IRS-2, which are essential for glucose homeostasis and  $\beta$  cell survival. Mice deficient in CREB activity in  $\beta$  cells develop diabetes secondary to  $\beta$  cell apoptosis [180], whereas overexpression of wild-type CREB in MIN6 cells protect against cytokine-induced apoptosis [177]. This reports point to CREB as an important target for strategies to improve  $\beta$  cell survival (**Fig. 1-47**).

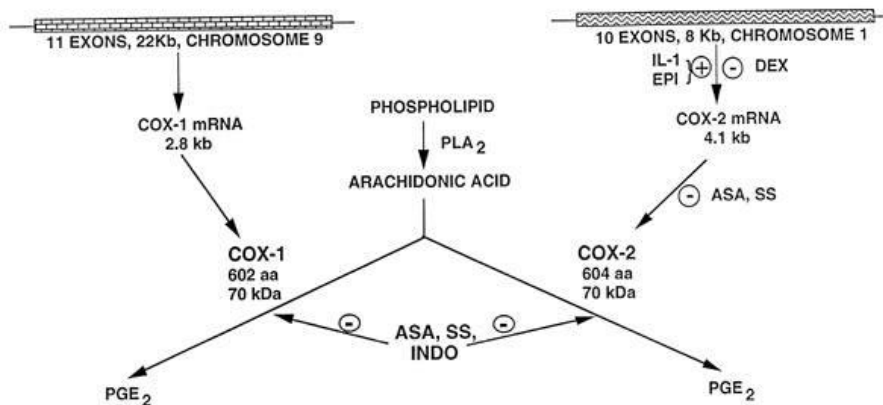


**Figure 1-47 CREB activation pathways**  
Source: Lifesciences

### 1.5.7.5.3 Inflammatory mediators implicated in NF- $\kappa$ B and CREB pathway

#### 1.5.7.5.3.1 Cyclooxygenase-2 (COX-2)

NF- $\kappa$ B and CREB are transcription factors implicated in COX-2 transcription. In response to inflammatory or stimulated events in tissues and specific inducers, phospholipase A<sub>2</sub> release arachidonic acid from cellular membrane phospholipids. COX-2 converts arachidonic acid to prostaglandin H<sub>2</sub> (PGE<sub>2</sub>), the common precursor to all prostaglandins, thromboxane and prostacyclins (**Fig 1-48**) [181].



**Figure 1-48 Molecular regulation of PG synthesis [181]**

Deregulation of signal transduction pathways (resulting from upregulation of COX-2 expression) by pro-inflammatory stimuli has been implicated in apoptosis inhibition, angiogenesis stimulus and promotes metastasis and immunosuppression [182].

COX-2 promoter contains the consensus CRE motif which is recognized by CREB. The CRE response element has been identified as one of the central regulatory elements in the COX-2 promoter region [183]. Activation of CREB by cAMP in response of the PGE<sub>2</sub> levels

regulates the DNA-binding activity of CREB to the CRE motif and subsequent COX-2 gene transcription [184]. COX-2 promoter also contains a NF- $\kappa$ B site located between 392 and 433 bp upstream of the transcription start site [185].

Hypoxia induces COX-2 expression in vascular endothelial cells mediated by NF- $\kappa$ B p65. The up-regulation of COX-2 in response to hypoxic stress is of pathophysiologic significance and might be a target for gene therapy. Blood in hypoxic vessels downstream from an arterial stenosis is known to be more prone to thrombosis. This could be related to an increased COX-2 production by hypoxic vascular endothelium and subsequent release of procoagulant thromboxanes [186].

Under physiological conditions, islets synthesized PGE<sub>2</sub>, a process known to be stimulated by glucose. Because PGE<sub>2</sub> is an inhibitor of glucose-insulin secretion modulating the adrenergic nervous system action that regulates insulin secretion, upregulation of COX-2 leads in PGE<sub>2</sub> overproduction, which plays a role in insulin release inhibition and develops hyperglycemia [187]. COX-2 stimulation by cytokines in rat and human islets leads to PGE<sub>2</sub> overproduction inducing  $\beta$  cell dysfunction and islet degeneration [188].

PGE<sub>2</sub> may be involved in stimulation of IL-6 synthesis. Addition of exogenous PGE<sub>2</sub> to macrophages induces IL-6 protein and mRNA synthesis, indicating that the eicosanoid stimulates IL-6 production at the level of gene expression [189]. The pro-inflammatory agents IL-1, TNF- $\alpha$  and LPS, as well as the growth factors TGF- $\beta$ , EGF, PDGF and FGF, have all been shown to induce COX-2. On the other hand, the anti-inflammatory cytokines IL-4 and IL-10, as well as the immunosuppressive glucocorticoids, were shown to decrease COX-2 levels [190, 191]. The rapid expansion of knowledge about the role of COX-2 in inflammation led to drug screens attempting to identify anti-inflammatory agents selective for COX-2 [192].

#### 1.5.7.5.3.2 Interleukin-6 (IL-6) and MCP-1

NF- $\kappa$ B and CREB may be implicated in IL-6 regulation through COX-2. Elevated PGE<sub>2</sub> from induction of COX-2 expression are involved in stimulating IL-6 synthesis *in vivo*. Additionally, reduces in NF- $\kappa$ B translocation was also correlated to reduces IL-6 and MCP-1 concentrations in islets culture medium [193].

Chemokines like IL-6 are described as inflammatory intermediaries in the infection sites or damage in response to pro-inflammatory stimulus. Inflammatory chemokines recruit and activate leucocytes in order to organize the immune response and initiate the healing [194]. IL-6 belongs to the pro-inflammatory cytokine family which are soluble proteins that allow the communication between the cell and the surrounded [195]. One of the main features of these molecules is their role in the immune system, implicated in cell traffic, tissue development and immune organs. The origin of the immune response determines the cytokine production and if the response is cytotoxic, humoral, cellular mediation or allergenic [196].

Inflammation and chemokine secretion has been described in islets [173]. In freshly isolated human islets, expression of cytokine IL-6 and MCP-1 are markedly elevated together with impaired insulin secretion function before transplantation [193]. Rat islets twelve hours after isolation showed an increase in IL-6 secretion [173]. Multiple pro-inflammatory cytokines including IL-6 were upregulated in pancreatic ischemia reperfusion injury, associated with pancreatic necrosis [197].

Monocyte chemoattractant protein-1 (MCP-1) is a member of the chemokine family and is produced by endothelial cells, vascular smooth muscle cells, keratinocytes, fibroblasts, mesangial cells, tubular epithelial cells, lymphocytes, and monocyte/macrophages in response to pro-inflammatory stimuli [198]. MCP-1 has been shown to have a variety of functions. *In vitro*, it is able to induce chemotaxis of monocytes at subnanomolar concentration [199].

MCP-1 expression could induce an intense inflammation and enhance subsequent specific immune response after transplantation. Expression of MCP-1 in human islets was recently shown to play an important role in the clinical outcome of islet transplantation in patients with type 1 diabetes. Patients who receive free islets that produce high levels of MCP-1 were not able to induce long-term insulin-independence. In contrast, patients who received free islets that produced low levels of MCP-1 reached long-lasting insulin independence. Other works recently reports high expression of MCP-1 mRNA and protein levels in rat and human islets cells exposed to cytokines [200].

Expression of these pro-inflammatory molecules could markedly influence the outcome of islet encapsulation and transplantation. New approaches to protect encapsulated islets from inflammation should be addressed for successful islet survival and function and long-lasting insulin independence.

## 2 OBJECTIVE

This work falls within the framework of BIOSID, a consortium funded by the European Commission (FP7, Grant agreement number HEALTH-F2-2012-30574) for the pre-clinical and clinical validation of the MAILPAN<sup>®</sup> as a bioartificial pancreas. In this consortium, the CEED was in charge of the optimization of cell survival and function for preclinical phases using the MAILPAN<sup>®</sup>.

As detailed above, the density of cells, conditioned by the need of insulin, could be detrimental for cell survival in an artificial environment in which oxygen availability is potentially low. Within the consortium, it has been shown that the partial pressure in oxygen of the MAILPAN<sup>®</sup> in vivo was around 15mmHg. Moreover, corresponding to the size of the device and the number of islets required to reverse diabetes, it has been established that the optimal density would be 600IEQ/cm<sup>2</sup>. Based on those first observations, survival rate of islet and mechanism triggered needed to be addressed.

- 1- The first step of the present work consisted in setting up an accurate *in vitro* model of the inner condition of the MAILPAN<sup>®</sup> and to understand in those conditions the cellular mechanisms responsible for early loss of islets.
- 2- Hypoxia being one of the limiting factors for islet survival, the aim of this part was to identify and study the impact of molecules able to provide oxygen on the hypoxic phenotype of islets.
- 3- Finally, inflammation markers identified in the first part as deleterious for islet survival were targeted with anti-inflammatory molecules.

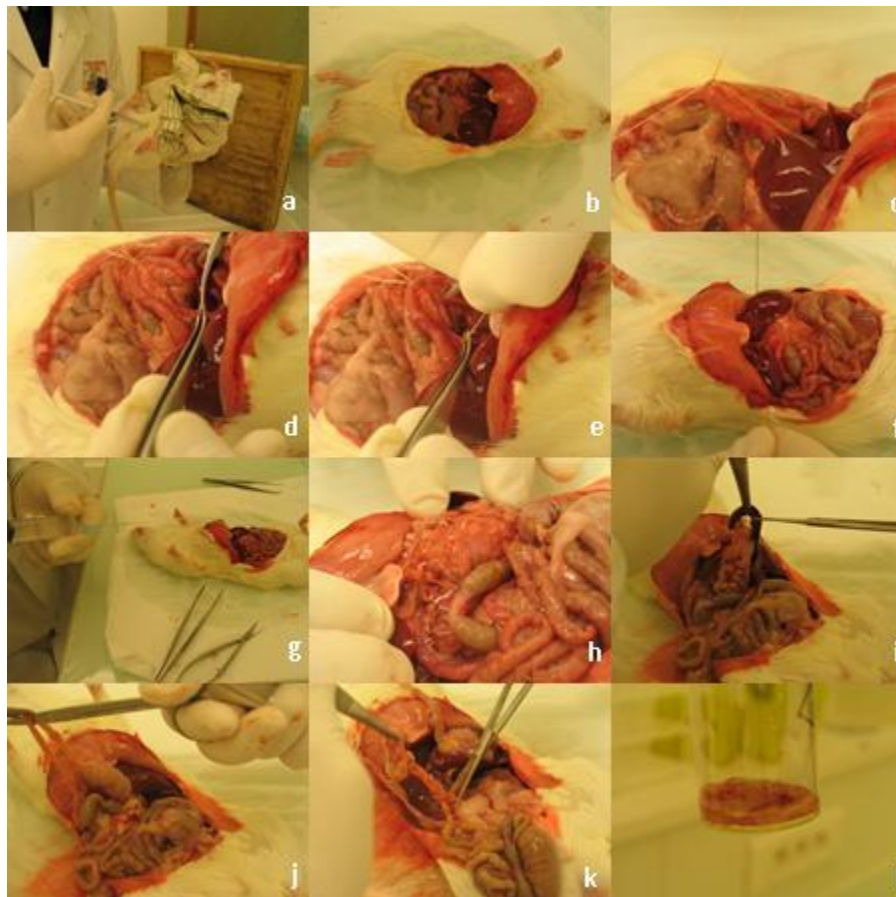
The final goal is to maintain islet viability for a longer term using the MAILPAN<sup>®</sup> in order to restore glycemic levels in type 1 diabetic patients without immunosuppressive treatment.

### 3 MATERIALS AND METHODES

#### 3.1 Pancreatic islets isolation

Les rats utilisés ont un poids corporel variant entre 200 et 250 g. Pour les études in vitro, les rats utilisés pour les isolements d'îlots pancréatiques sont des rats Wistar.

Les rats sont anesthésiés par l'injection intrapéritonéale d'un mélange d'Imalgene (Merial, Lyon, France) + Rompun (Bayer, France) (2,7ml Rompun pour 10ml d'Imalgene) à raison de 100  $\mu$ l/100 g de poids corporel. Après laparotomie, le pancréas est dégagé, le canal cholédoque est ligaturé à l'embouchure duodénale et cathétérisé à l'embouchure hépatique. Dix ml de collagénase de *Clostridium histolyticum* (type XI, Sigma-Aldrich) sont injectés dans le pancréas et l'animal est sacrifié par exsanguination pour le prélèvement de l'organe. Les pancréas sont conservés à 4°C dans 7,5 ml de solution de perfusion (0,035% de bicarbonate de sodium (Sigma-Aldrich), 2,35 mmol.l<sup>-1</sup> de chlorure de calcium (Sigma-Aldrich), 0,025 mmol.l<sup>-1</sup> d'HEPES (Fisher, Illkirch, France) dilués dans de l'HBSS) (**Fig. 3-1**).



**Figure 3-1 Rat pancreas isolation**

a- Anesthésie du Rat, b- laparotomie, c- Ligature du canal cholédoque à l'embouchure duodénale, d- incision du canal cholédoque, e- Insertion du cathéter dans le canal Cholédoque, f- Fixation du cathéter dans le canal cholédoque jusqu'à l'embouchure duodénale, g- injection de collagénase h- dilatation du pancréas, i- dégagement du pancréas au niveau de la rate, j- dégagement du pancréas au niveau du duodénum, k- dégagement du pancréas au niveau de l'intestin, l- isolement du pancréas.



Les pancréas sont ensuite digérés sous agitation à 37°C au bain marie pendant 10 minutes. La digestion est stoppée avec 40 ml de milieu de rinçage à 4°C (milieu M199 supplémenté avec 10% de SVF décomplémenté et 5% AB/AM). Le digestat obtenu est filtré à travers un tamis (porosité de 500 µm, Sigma-Aldrich), rincé avec le milieu de rinçage puis transféré dans des tubes de 200 ml (Dutscher, Brumath, France). Le filtrat est centrifugé à 4°C pendant 1 minute à 300 x g. Les culots sont suspendus dans du milieu de rinçage, transférés dans un tube à centrifuger de 50 ml et centrifugés à 4°C pendant 1 minute à 300 x g.

Le surnageant est éliminé et les îlots sont séparés du tissu exocrine à l'aide d'un gradient discontinu de ficoll (Ficoll®PM 400, Dutscher), préparé au laboratoire et constitué de 3 densités (1,110 - 1,096 - 1,069) auxquelles se superpose 5 ml de HBSS. Ce gradient est centrifugé pendant 4 minutes à 30 x g sans accélération ni frein et 12 minutes à 800 x g. Le gradient est alors constitué de 4 phases : la majorité des îlots se concentre dans les phases 1 et 2, la 3ème phase a une pureté en îlots estimée à 20%, la 4ème phase et le culot contiennent essentiellement du tissu exocrine. Enfin, les phases 1 et 2 sont lavées 3 fois avec du milieu de rinçage par centrifugation (2 minutes à 300 x g) afin d'éliminer le ficoll. Les îlots sont ensuite mis en culture dans flasques de Medium 25 cm<sup>2</sup> de milieu M199 (Cellstar, Greiner Bio One GmbH, Frickenhausen, Allemagne) supplémenté avec 10% de SVF et 5% d'AB/AM pendant 24 heures avant de débiter toute expérience.

## 3.2 Culture d'îlots pancréatiques

### 3.2.1 Densité d'ensemencement

Après une culture de 24 heures, les îlots ont été comptés puis ensemencés dans des plaques 48 puits (Greiner) à raison de 150, 300 and 600 IEQ/cm<sup>2</sup> puis mis en culture pendant 24 h soit sous atmosphère normale (PO<sub>2</sub> :160mmHg) soit en conditions hypoxiques (PO<sub>2</sub>: 15 mmHg) dans une chambre d'hypoxie (StemCell, Technologies, Canada) contenant un mélange de 2%O<sub>2</sub>, 5%CO<sub>2</sub> and 93%N<sub>2</sub> (**Fig. 3-2**).



**Figure 3-2 Hypoxic chamber**

### 3.2.2 Culture d'îlots en présence de molécules

Une densité de 600IEQ/cm<sup>2</sup> a été utilisée pour tester les différentes molécules. 600IEQ/cm<sup>2</sup> ont été mis en culture dans des plaques à 48 puits pendant 24 heures sous hypoxie en présence de molécules. Afin d'améliorer la survie et la fonction de l'îlot, PFD, HEMOXCell<sup>®</sup> Ibuprofen, IL-10 et tat-CREB peptide ont été testés.

L'Ibuprofène est un médicament anti-inflammatoire non-stéroïdien (NSAIDs), utilisé en tant qu'inhibiteur COX-2 afin d'étudier la relation potentielle entre inflammation et cancer [201]. L'Ibuprofène est en compétition avec l'acide arachidonique pour la liaison avec le site actif

COX-2 et inhibe la production de PGE<sub>2</sub>. Il est utilisé pour apaiser la douleur, la fièvre et réduire les inflammations.

Le peptide tat-CREB est un dérivé de HIV-1, qui permet une délivrance efficace de la protéine ou d'un peptide dans les cellules par les membranes du plasma, et d'un acide peptide 10-amino dérivé de la séquence de ciblage proteosomal CREB. Il a été montré que cette peptide pénètre dans les cellules et se localise dans le cytoplasme ainsi que dans le nucléus et nucléole. La fonction du peptide est l'inhibition de la dégradation CREB par voie ubiquitine-protéasome [202]. CREB est également essentiel pour l'homéostasie du glucose et la survie des cellules  $\beta$ . Les hyperglycémies chroniques sont reconnues pour avoir des effets secondaires sur la survie et la fonction des cellules  $\beta$  par une réduction de la sécrétion d'insuline en réponse au stockage de glucose et d'insuline, appelé glucotoxicité. Des études antérieures ont démontré que l'utilisation du peptide tat-CREB sur des îlots humains exposés à une concentration de glucose élevée bloquaient la dégradation de CREB avec une préservation de la détection du glucose, une transcription du gène insuline et la sécrétion d'insuline [202]. Taylor *et al.* a démontré que l'addition de tat-CREB permettait de prévenir la dégradation de CREB provoquée par l'hypoxie [178]. The use of tat-CREB peptide has never been testing before in protects islets against inflammation. Le peptide tat-CREB n'avait jamais été testé sur la protection des îlots contre l'inflammation alors que CREB est connu pour réguler certains gènes impliqués dans la réponse pro-inflammatoire [184].

IL – 10 est une cytokine antiinflammatoire. IL-10 inhibe la production d'IL-6 et l'expression de la HLA-DR par des monocytes humains. IL-10 peut être exprimé par une variété de cellules, généralement en réponse à l'activation d'un stimulus ; son expression est régulée par différents mécanismes dans plusieurs types de cellules, comme les cellules T, les monocytes/macrophages. Les effets inhibiteurs de l'IL-10 sur la production d'IL-6 sont cruciaux pour la réponse anti-inflammatoire, car ces cytokines ont des activités synergétiques sur les voies inflammatoires et les processus, et il amplifie ces réponses en induisant les médiateurs secondaires comme les chemokines et les prostaglandines [201]. Pour le diabète de type 1, la perte progressive de cellules  $\beta$  produit la libération de cytokines anti-inflammatoires, en lien avec la protection des cellules  $\beta$  [203]. Certaines expériences supportent le rôle prévisible de l'IL-10 comme un facteur de protection face au diabète. Un traitement journalier sous-cutané de 9-10 semaines NOD chez les souris asymptomatiques retarde l'apparition du diabète et réduit les incidences de la maladie. Le traitement avec l'IL-10 a également réduit une insulite sévère et prévient l'infiltration cellulaire des cellules des îlots pancréatiques [204]. IL-10 inhibe également la production de prostaglandines PGE<sub>2</sub>, par la diminution de la régulation des taux des ARN messagers de COX-2 [191]. En réponse aux stimuli *in vitro*, IL-10 inhibe l'activation de NF- $\kappa$ B de deux manières différentes : en inhibant l'activation de l'I $\kappa$ B Kinase—similaire au salicylate et en liant l'activité de fixation de NF- $\kappa$ B sur l'ADN [201]. La libération de IL-10 est liée à la protection de cellules  $\beta$  et la prévention insulite destructrice [203]. Pendant les inflammations aiguës les effets inhibiteurs de l'IL-10 sur la production de cytokine pro-inflammatoire et la physiologie des types de cellules individuelles suggère qu'il pourrait avoir des activités anti-inflammatoires potentielles *in vivo*.

### 3.2.2.1 Préparation de milieu avec différentes molécules

#### - Perfluorodécane (PFD):

Le milieu de culture a été supplémenté avec 10% de PFC (F2 Chemicals Ltd., Lancashire, UK). Le Milieu a été saturé en oxygène avant culture

- HEMOXcell<sup>®</sup>:

HEMOXcell<sup>®</sup> a été utilisé à une concentration de 50µg/L. Le milieu final été saturé en oxygène avant culture.

- Tat-CREB peptide:

Tat-CREB peptide (1mg/ml in DMSO) a été dilué dans le milieu de culture à une concentration finale de 10 µM.

- Ibuprofen:

Le milieu de culture a été supplémenté avec de l'Ibuprofène (SIGMA) à une concentration finale de 1mM.

- IL-10:

Le milieu de culture a été supplémenté avec de l'IL-10 à une concentration finale de 10 ng/ml.

### **3.3 Viabilité des îlots pancréatiques et tests fonctionnels**

#### **3.3.1 Viabilité des îlots**

La viabilité des îlots de chaque condition après une culture de 24 heures sous condition normoxique ou hypoxique a été analysée avec le test à la fluorescéine de diacétate avec coloration à la fluorescéine iodure de propidium (FDA/PI, Sigma) ou avec LIVE/DEAD<sup>®</sup> Kit de viabilité/cytotoxicité (ThermoFisher Scientific, Illkirch-Graffenstaden, France) en suivant les instructions du fabricant par trois chercheurs indépendants.

FDA (esters non-polaires) traverse le plasma des cellules vivantes puis il est hydrolysé par les esters intracellulaires. L'hydrolyse FDA produit une synthèse de fluorescéine polaire qui reste à l'intérieur des cellules avec une membrane de plasma intacte. Par conséquent les cellules vivantes sont vert fluorescent détectables par microscopie à fluorescence. Le PI traverse les membranes des cellules nécrotiques ou mortes. A l'intérieur des cellules, le PI est fixé à l'acide nucléique qui est rouge fluorescent. L'utilisation de FDA (0,67 µmol.l-1) et de PI (4 µmol.l-1) en même temps, a permis de différencier les cellules vivantes des cellules mortes.

LIVE/DEAD<sup>®</sup> Kit de viabilité/cytotoxicité utilise un colorant poly anionique calceine AM qui est retenu à l'intérieur des cellules produisant du vert fluorescent pour l'analyse les cellules vivantes et EthD1 qui pénètre avec des dommages sur les membranes et elles sont liées aux acides nucléiques produisant une fluorescence rouge chez les cellules mortes ce qui permet de les analyser.

Pour ces deux méthodes la viabilité des îlots pancréatique est estimée visuellement et représentée en pourcentage de cellules mortes ou vivantes pour un même îlot. Le ratio de cellules vertes à rouges détermine le pourcentage de viabilité de l'îlot. Les images obtenues proviennent d'un microscope Nikon Eclipse 50i et du logiciel Nis-Element-BR (Nikon, Amstelveen, Pays-Bas).

A fin de vérifier les résultats obtenus avec les méthodes décrites précédemment, l'activité des marqueurs apoptotiques caspase 3 et 7 ont été mesurés en utilisant le test Caspase-Glo<sup>®</sup> 3/7 (Promega) en respectant les instructions du fabricant.

### 3.3.2 Fonctionnalité des îlots

#### 3.3.2.1 Niveaux des ATP

Un total de 50 îlots pour chaque condition a été utilisé pour l'extraction de l'ATP. L'ATP est un indicateur de fonctionnalité des cellules, car il est présent dans toutes les cellules métaboliquement actives et la concentration décline assez rapidement lorsque les cellules subissent une nécrose ou apoptose. Le rajout de luciférase et D-Luciferin réagit avec l'ATP. La lumière émise est proportionnelle à la concentration d'ATP. Les niveaux d'ATP ont été évalués en utilisant un Kit ATPLite (PerkinElmer, Inc., Waltham, MA, USA) en suivant les instructions du fabricant.

#### 3.3.2.2 Test de sécrétion d'insuline au cours d'une stimulation au glucose

La fonctionnalité des îlots pancréatiques est évaluée à l'aide d'un test de stimulation au glucose. Ce test permet d'évaluer la quantité d'insuline sécrétée par les îlots pancréatiques après leur stimulation avec une solution riche en glucose. Un sous-ensemble d'îlots (n=10) de chaque condition expérimentale ont été lavés et incubés dans une solution de bicarbonate Kreb's Ringer (KRB) avec 10% de FBS et 4.4 mmol/L de glucose (SIGMA). Les cellules sont ensuite stimulées avec la solution KRB contenant 10% de FBS et 22.5mmol/L de glucose et pour la dernière étape les îlots ont été incubés à nouveau dans une solution KRB contenant 10% de FBS et 4.4 mmo/L de glucose. Chaque étape d'incubation a été réalisée pendant 90 minutes à 37° en atmosphère humide enrichie de 5% de CO<sup>2</sup>. Les surnageants ont alors été prélevés et stockés à -80°. L'insuline présente dans les surnageants prélevés est ensuite dosée afin de comparer la quantité d'insuline sécrétée par les îlots en condition basale et stimulée. Le dosage de l'insuline se fait à l'aide d'un test de dosage immunoenzymatique "Enzyme Linked Immunosorbent Assay" (ELISA): Mercodia Rat insulin Elisa (Enzyme Linked Immunosorbent Assay, Mercodia, Uppsala, Suède) (Voir chapitre 3.5.3).

L'insuline contenue a été normalisée par la quantité de protéines extraite en fin de stimulation et est exprimée en µg/mg de protéine et la fonctionnalité est exprimée selon l'index de stimulation (SI) calculé par le rapport entre l'insuline sécrétée en présence de 22.5mmol/L de glucose et l'insuline sécrétée en présence de 4.4mmol/L de glucose.

### 3.4 Chimiotactisme

Les macrophages sont activés et isolés chez des rats Wistar mâles d'environ 300 g (Elevage Depré, St Doulicher, France). Pour cela, une péritonite chimique est induite par injection intra-péritonéale de 10 ml d'une solution de thioglycolate (Sigma-Aldrich) à 3%. Le prélèvement est réalisé 72 heures après l'injection. L'animal est anesthésié par l'injection intrapéritonéale d'un mélange d'Imalgène<sup>®</sup> (Merial, Lyon, France) + (Rompun<sup>®</sup>, Bayer, Puteaux, France) (2,7ml de Rompun pour 10ml d'Imalgène) à raison de 100 µl/100 g de poids corporel puis décapité pour une exsanguination totale.

Les cellules péritonéales sont ensuite prélevées, après laparotomie, par des lavages péritonéaux successifs avec 50 ml de « Phosphate Buffered Saline » (PBS ; Sigma-Aldrich) contenant 1% d'ABAM.

Après recueil des cellules péritonéales, ces dernières sont centrifugées 5 minutes à 400g et reprises dans le volume requis de milieu M199 supplémenté (Sigma-Aldrich) avec 10% de SVF et 1% d'ABAM.

L'étude du chimiotactisme est réalisée à l'aide d'une chambre de Boyden modifiée dont le principe repose sur la migration cellulaire induite par la présence d'un gradient chimiotactique entre deux compartiments. La chambre de Boyden est composée de deux chambres séparées par un filtre à travers lequel les cellules migrent.

Un insert (Dutscher) avec une membrane de polycarbonate d'une porosité de 8 µm est placé dans chaque puits d'une plaque 24 puits (Dutscher). Les macrophages sont ensuite placés dans le compartiment du haut à raison de 500 000 macrophages par insert dilués dans 200 µl de milieu M199 en présence d'un traitement ou pas. Le compartiment du bas contient 400 µl de solution chimiotactique à tester. Le formyl-Met-Leu-Phe (fMLP ; Sigma), reconnu pour son fort pouvoir chimio attractant, est utilisé comme contrôle positif de migration. Le fMLP est un tripeptide de synthèse, analogue de produits du métabolisme bactérien. Le milieu M199 est utilisé comme contrôle négatif de la migration macrophagique. Après 4 heures d'incubation à 37°C en atmosphère humide enrichie de 5% de CO<sub>2</sub>, le chimiotactisme est évalué après fixation et coloration (Diff-Quick®, Dade Behring, Courbevoie, France) des macrophages accolés à la membrane du côté extérieur de l'insert. Après séchage à température ambiante, s'ensuit une décoloration à l'acide chlorhydrique (VWR, Strasbourg, France) à 0,1 mol.l<sup>-1</sup>. La lecture se fait par spectrophotométrie à 605 nm. Le chimiotactisme est évalué par le calcul de l'index de migration:

$$\frac{\sum_{i=1}^n \text{O.D.}_{605}(\text{cellules}) \times h_{\text{cellules}}}{\sum_{i=1}^n \text{O.D.}_{605}(\text{contrôle}) \times h_{\text{contrôle}}} \pm \text{écart type}$$

### 3.5 Extraction et dosage des protéines

#### 3.5.1 Extraction

Après 24 heures de traitement, les îlots pancréatiques ont été lavés avec le PBS puis centrifugés

Pendant 2 minutes à 300g. La totalité de l'extraction a été réalisé avec M-PER (M-PER Mammalian Protein Extraction Reagent, et un cocktail inhibiteur de Phosphatase HALT™ (ThermoFisher Scientific, Illkirch-Graffenstaden, France).

Le tampon de lyse est supplémenté avec un cocktail d'inhibiteurs de protéases. Après incubation pendant 10 minutes sur la glace, les îlots sont centrifugés à 14.000g pendant 15 minutes. Les surnageants (extrait de protéines) sont collectés et conservés à -80°C.

#### 3.5.2 Mesure de protéine

La concentration de protéine a été déterminé en utilisant la micro-méthode de Bradford (Bio-Rad, Life Science Group, Marnes-la-Coquette, France). Cette méthode est un dosage colorimétrique, basée dans les échanges absorption. Ce changement est dû au bleu de Coomassie, qui change de couleur en se liant avec des acides aminés aromatiques (tryptophan, tyrosine et phenylalanine) et des résidus d'acides aminés présents ou des protéines. La coloration du bleu de Coomassie est proportionnelle à la quantité de protéines. 5 µg de protéine ont été déposés dans une plaque à 96 puits et additionné de 250 µg de solution Bradford. Après 10 minutes d'agitation délicate dans le noir, l'absorbance de la plaque a été mesurée at 595nm. On calcule ensuite la concentration de protéine en comparant l'échantillonnage standard de BSA préparé sous les mêmes conditions. Les concentrations de protéines ont été exprimées en mg/mL.

### 3.5.3 ELISA

Un ELISA utilise un anticorps spécifique de la protéine d'intérêt coaté sur une plaque à 96 puits. Les extraits protéiques sont incubés dans les puits puis retirés. Grâce aux anticorps, les protéines d'intérêt sont fixées dans la plaque. Après lavage, les anticorps biotinylés anti-protéines d'intérêt sont rajoutés. Après le lavage permettant de supprimer les anticorps biotinylés non fixés, le HRP- streptavidine conjugué est ajoutée dans le puit. Le conjugué HRP-Streptavidine est détecté par réaction avec le substrat 3,3'-5,5'-tetramethylbenzidine (TMB). La réaction est stoppée par l'addition d'acide, donnant ainsi un changement de couleur mesuré avec un spectrophotomètre à une longueur d'onde de 450 nm. La quantité de protéine d'intérêt peut être déduite grâce aux standards.

Les différents kits ELISA ont été utilisés selon les recommandations du fournisseur et les références sont regroupées dans le tableau 3-1:

Antigène	Reference	Supplier
<b>IL-6</b>	Rat IL-6 Quantikine ELISA Kit	R&D Systems®
<b>MCP-1</b>	Rat MCP1 ELISA Kit	RayBiotech®, Inc., Clinisciences
<b>Caspase-3</b>	Human Active Caspase-3 Quantikine® ELISA	R&D Systems®
<b>VEGF</b>	Rat VEGF ELISA KIT	RayBiotech®, Inc., Clinisciences
<b>Insulin</b>	Rat Insulin ELISA	Mercodia
<b>NF-κB p65*</b>	TransAM™ NF-κB	Active Motif
<b>HIF-1α*</b>	TransAM™ HIF-1	Active Motif
<b>CREB*</b>	TransAM™ CREB	Active Motif
<b>pCREB*</b>	TransAM™ pCREB	Active Motif
<b>Prostaglandins</b>	Prostaglandin E <sub>2</sub>	R&D Systems®

**Tableau 3-1 Listes des Kits ELISA utilisés**

\* Les Kits TransAM™ sont des DNA-liant ELISAs

### 3.5.4 Western Blot

L'extrait protéique (20 µg de protéines) mélangé avec des tampons échantillons XT 1X (Bio-Rad, France) est dénaturé à 95°C et déposé sur un gel de polyacrylamide 12% (Criterion™ XT 4-12%Bis Tris, Bio-Rad). Les protéines sont séparées par électrophorèse (200 Volts pendant 40 minutes).

**Après 5 minutes le transfert humide est réalisé sur membrane de Polyvinylidene Difluoride pendant 40 minutes à 100 Volts. Après transfert, la membrane est saturée 1 heure sous agitation à température ambiante dans du TBS (TBS-T) supplémenté avec 0,1 % de Tween 20 (Sigma-Aldrich) et 5% de BSA (Sigma-Aldrich). La membrane est ensuite incubée la nuit à 4°C avec l'anticorps primaire à la dilution optimale dans le tampon de blocage (Tableau 3-2). La membrane est ensuite lavée trois fois pendant 5 minutes sous agitation dans le TBS-T, puis incubée pendant 1 heure à température ambiante avec l'anticorps secondaire correspondant couplé à la peroxydase et dilué dans le tampon de blocage. Après trois lavages avec le TBS-T, le complexe d'anticorps est révélé par chimioluminescence avec un kit de détection (Immun-Star™ Western**

C<sup>TM</sup>, Bio-Rad). L'imageur Chemidoc XRS (Bio-Rad) permet l'acquisition du signal de chimioluminescence pour une exposition optimale des membranes, et une capture par caméra à transfert de charge (CCD) refroidie. L'exploitation numérique de l'image obtenue est réalisée à l'aide du logiciel Image J.

<b>Primary antibody</b>				
<b>Antigen</b>	Antibody	References	Supplier	Dilution
<b>Rat- COX2</b>	Polyclonal-Rabbit	ab15191	Abcam <sup>®</sup>	1/1000
<b>Rat- Phospho-NFκB p65</b>	Monoclonal-Mouse	13346	Cell Signalling <sup>®</sup>	1/500
<b>Rat- NFκB p65</b>	Monoclonal-Rabbit	8242	Cell Signalling <sup>®</sup>	1/500
<b>Rat- βactine</b>	Monoclonal-Mouse	Sc-32251	Santa Cruz	1/2000
<b>Rat- βactine</b>	Monoclonal-Rabbit	ab1801	Abcam <sup>®</sup>	1/2000
<b>Secondary antibody</b>				
<b>HRP</b>	Anti-Mouse	A0545	Sigma	1/5000
<b>HRP</b>	Anti-Rabbit	A9044	Sigma	1/5000

**Table 3-2 Liste des anticorps utilisés avec le Western Blot**

## **3.6 L'expression du Gène**

### **3.6.1 Extraction d'ARN**

L'ARN total est extrait des cellules en culture à l'aide du Kit RNeasy Plus Mini (Qiagen, CA, USA) selon le protocole défini par le fournisseur. Le tampon RLT plus est une solution mono-phasique de phénol et d'isothiocyanate de guanidine qui permet de lyser les cellules et de dissoudre les composants cellulaires tout en préservant l'intégrité des ARNs.

Les cellules sont lysées dans 350 µl de tampon RLT plus. Le lysat est ensuite homogénéisé à l'aide d'une seringue munie d'une aiguille afin de réduire la viscosité et donc de favoriser la fixation de l'ARN dans la colonne d'extraction. Le lysat est transféré dans une première colonne et centrifugé à 8000 x g pendant 30 secondes afin d'éliminer l'ADN génomique. Puis, les ARNs contenus dans le filtrat sont précipités par addition de 350 µl d'éthanol 70%. Le mélange est transféré dans une deuxième colonne qui retient l'ARN puis centrifugé à 8000 x g pendant 15 secondes. Puis la colonne est lavée avec 700 µl de tampon RW1 et centrifugée à 8000 x g pendant 15 secondes. La colonne est de nouveau lavée deux fois avec 500 µl de tampon RPE puis centrifugée à 8000 x g pendant 15 secondes et pendant 2 minutes pour le dernier lavage. Afin de s'assurer de l'élimination de tous les tampons de lavage utilisés, la colonne est centrifugée seule à 20 000 x g pendant 1 minute. Enfin, l'ARN est solubilisé avec 20 µl d'eau ARNase free. Les concentrations sont déterminées par densité optique à 260 nm et la pureté des échantillons d'ARN est calculée par le rapport des densités optiques à 260 et 280 nm. Un rapport égal ou supérieur à 1,8 indique l'absence de contamination de la préparation d'ARN par les protéines.

### 3.6.2 Transcription inverse d'ARN

L'obtention d'ADNc totaux est réalisée par transcription inverse (RT) sur 1 µg ou 500 ng d'ARN total à l'aide du kit « RT2 First Strand Kit » (SABiosciences™, Qiagen) suivant les instructions du fabricant. Une précaution est prise afin d'éliminer l'ADN génomique, 8 µl d'ARNs sont incubés pendant 5 min à 47°C avec 6 µl de tampon d'élimination d'ADN génomique. Ensuite 6 µl de tampon BC4 de Transcriptase inversé a été rajouté au volume final de 20 µl. Les échantillons sont incubés avec un programme à (42°C pendant 15 min, 95° 5 minutes, 4° maintien) pour réaliser la transcriptase inverse.

### 3.6.3 Quantification de l'ARN Real-time PCR

Les ADNc spécifiques des ARNm étudiés sont amplifiés en utilisant le kit « QuantiTect® SYBR® Green PCR » (Qiagen) sur un système PCR MyiQ Real-Time (Quagen). Des courbes de standardisation sont obtenues pour chaque gène étudié (**Tableau 3-3**), par dilutions en cascade d'un pool d'échantillons contrôle d'ADNc. Le milieu réactionnel (25 µl) contient 12,5 µl de « 2x Quantitect SYBR Green PCR Master Mix » (Qiagen), 2,5 µmol de chaque couple d'amorces sens-antisens spécifiques de chaque gène étudié, 10 µl d'ADNc (dilué au 1/100 d'ARN rétrotranscrit), selon les recommandations du fournisseur. Après une première dénaturation à 95°C pendant 15 min, la RT-PCR est réalisée en 35 cycles de dénaturation (15 sec à 94°C), d'hybridation (30 sec à 55°C) et de polymérisation (30 sec à 72°C). L'établissement d'une courbe des points de fusion est systématiquement réalisé suivant les instructions proposées par le fournisseur du logiciel du thermocycleur (IQ™5, Bio-Rad). Cette courbe permet de vérifier la spécificité des produits de RT-PCR. Un contrôle négatif est systématiquement réalisé en remplaçant l'ADNc par de l'eau stérile de qualité PCR. Chaque échantillon est analysé en doublet et quantifié avec le logiciel d'analyse du thermocycleur.

L'expression relative des différents gènes est calculée en rapportant les taux d'ARNm à ceux de 3 gènes d'intérêts différents en utilisant la méthode  $\Delta\Delta Ct$ .

Primers	Reference
Rn_Rnr1_1_SG QuantiTect primer assay	QT00199374
Rn_Ppia_1_SG QuantiTect primer assay	QT00177394
Rn_Rplp1_2_SG QuantiTect primer	QT01745625
Rn_Hif1a_1_SG QuantiTect primer assay	QT00182532
Rn_Ptgs2_1_SG QuantiTect primer assay	QT00192934
Rn_Hmox1_1_SG QuantiTect Primer Assay	QT00175994

**Table 3-3 Liste des primers utilisés pour le PCR**



### 3.7 Mesures d'oxygène

Les mesures d'oxygène ont été réalisées à l'aide d'un mètre (Oxy-4) avec une fibre optique à quatre canaux et des senseurs d'oxygène non-invasifs (Spots, Presens, Allemagne). Les spots ont été collés en bas de chaque plaque de culture (Presens, Allemagne), permettant une mesure non-invasive de l'oxygène pouvant être prise à travers le plastique depuis l'extérieur. Une fibre optique connectée au mètre d'oxygène OXY4 a été positionnée par l'extérieur de la chambre vers le spot. Les données sont exprimées en mmHg et relevés au temps 0 (t0) avant de placer les plaques dans les incubateurs et également après la période d'incubation de 24h.

### 3.8 Histologie

Les îlots pancréatiques ont été récoltés, lavés et surgelés dans un composite à température optimale de découpage (Tissue O.C.T. Labonor, Templemars, France) dans un moule d'inclusion (Leica, France). Les moules d'inclusion ont été stockés à -80°. Les sections ont été réalisées dans le cryostat (Leica CM30550S, Leica, France) à 12 µm de section. Les sections ont été récupérées sur les lames SuperFrost Plus (Labonor) et stockées à -80°.

Les lames ont été décongelées à température ambiante pendant l'immunohistochimie et maintenu/fixé avec de la paraformaldehyde (PFA) 4% (Sigma) pendant 10 minutes. Après, les lames ont été lavées dans de la solution saline tamponnée au tris (TBS, Bio-Rad). Pour activer les peroxydases endogènes, les lames ont été incubées dans une solution d'hydrogène peroxydase 3% (Sigma) pendant 10 minutes. Les sites spécifiques ont été bloqués pendant une heure à température ambiante avec de la solution bloquante contenant du TBS 0.5%, Triton – X 100 (Sigma) et 5% de sérum de chèvre (Thermo Fisher, France). Puis les lames ont été incubées sur la nuit à 4°C dans une chambre humide avec le premier anticorps dilué dans une solution bloquante. Après incubation, les lames ont été lavées PBS 1X et incubées pendant 1 heure à température ambiante dans une chambre humide avec le deuxième anticorps lié à un composé fluorescent puis dilué dans une solution bloquante. Après 1 heure, les lames ont été rincées et préparées dans un milieu de montage (Vectashield, Clinisciences) de l'ADN liant réactif comme 4'-6'-diamino-2-phenylindole 'DAPI, Vectashield, Clinisciences) a été rajouté dans le milieu de montage. Les lames ont été observées par microscopie à fluorescence.

Le marquage de l'apoptose a été réalisé en utilisant un Terminal deoxynucleotidyl transférase dUTP Nick End Labelling (TUNEL) assay (Millipore, Molsheim, France) en suivant les instructions du manuel. Le marquage de l'hypoxie a été réalisé en utilisant un Hypoxyprobe TM-1 Kit (HPI, Inc. Burlington, MA, USA). Dans cette étude, de la pimonidazole HCl solide, qui est un marqueur hypoxique, a été rajouté pendant la culture d'îlots avec une concentration de 150 µM. Les anticorps utilisés dans cette étude ont inclus de l'anti-mousse immunoglobulin (IgG1, 1:50 dilution; HPI, Inc.) et de l'Alexa Fluor 488 anti-mousse de chèvre IgG (488 (dilution 1:200; Life technologies, Carlsbad, CA, USA).

### 3.9 Analyse statistique

Des analyses statistiques ont été réalisées en utilisant le logiciel Statistica (StatSoft, Maisons-Alfort, France). Les résultats ont été analysés avec des analyses unidirectionnelles

de variances (ANOVA) suivi par un test de Tukey avec une différence significative (HSD) pour les données paramétriques ou Kruskal-Wallis pour les analyses non-paramétriques. Les résultats sont exprimés en moyenne  $\pm$  SEM. Une valeur p inférieure à 0.05 était considérée statistiquement significative.

## 4 RESULTS

### 4.1 Islet confinement under hypoxia

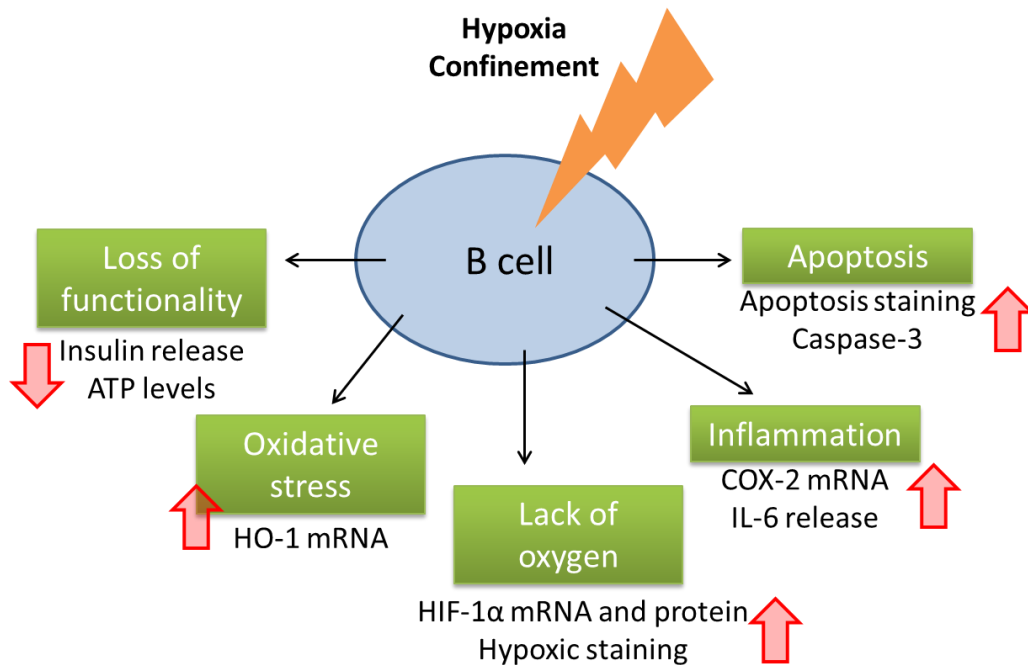
Encapsulation of islet in the bioartificial pancreas is one of the strategies to avoid the harmful consequences produced by the immunosuppressive treatment a long-term. However, as an artificial environment, the survival of cells could be impaired. Indeed, the reduced space and the low oxygenation could have an impact on cell.

The number of cells per square centimeters had been studied for one type of BP, however, the characteristics of our device is different. Thus the study of rate survival in the condition *in vitro* of our models was necessary.

First we looked at the concentration of oxygen in the device. A spot was implanted in the device and 30 days after implantation, the measure was performed and it was found that 15 mmHg was the partial pressure in the device. Moreover, the calculation of the islet requirement for diabetes reversion stated that 10,000 IEQ/device are needed which corresponds in our case to 600 IEQ/cm<sup>2</sup>. Therefore we set our experiment to be as closed as possible to the *in vivo* conditions.

The effect of different densities had been tested under normoxia and hypoxia on islets. We have shown that the combination of hypoxia and increased islet density/confinement, which are the typical conditions in the bioartificial pancreas, causes an increased level of islet cell death. Indeed, when the recommended culture density (150IEQ/cm<sup>2</sup>) was doubled (300IEQ/cm<sup>2</sup>) or multiplied by four (600IEQ/cm<sup>2</sup>) and maintained under hypoxic conditions, the overall survival of the islets was very poor, with an observed activation of apoptosis and necrosis factors as well as loss of function and pro-inflammatory cytokine secretion. Islets in the bioartificial pancreas will never be vascularized due to the immune-isolation required. Therefore, no oxygen or nutrients will be delivered directly to islets by the vessels, because only the device can be vascularized, resulting in a low cellular PO<sub>2</sub> (around 15 mmHg). Our results showed that when the number of islets was doubled or increased four times, the oxygen concentration dropped from 15 mmHg to 7 mmHg having repercussions in terms of islet survival. Moreover, under hypoxic conditions, we observed the effects of cell density to be amplified, leading to cell death and loss of function. We have shown that hypoxic conditions combined with confinement triggered HIF-1 $\alpha$  transcription, which is correlated with activation of COX-2 and HO-1 mRNA expression. These data imply that the cells are at least attempting to activate their endogenous defense system against the lack of oxygen. Decreased in cellular ATP content was also enhance under hypoxia due to the ATP leakage

and correlated with a decrease in cell's viability to respond to glucose stimulation. IL-6 secretion appears to be related to confinement only. The more cells there are in a limited space, the more IL-6 will be secreted as each islet will be more able to induce the surrounding cells (**Fig. 4-1**).



**Figure 4-1 Summary of the issues in encapsulated islets**

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## **Impact of pancreatic rat islet density on cell survival during hypoxia**

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## **ABSTRACT**

In bioartificial pancreases (BP), the number of islets needed to restore normoglycaemia in the diabetic patient is critical. However, the confinement of a high quantity of islets in a limited space may impact islet survival, particularly in regards to the low oxygen partial pressure ( $PO_2$ ) in such environments. The aim of the present study was to evaluate the impact of islet number in a confined space under hypoxia on cell survival. Rat islets were seeded at three different concentrations (150, 300, and 600 Islet Equivalent (IEQ)/ $cm^2$ ) and cultured in normal atmospheric pressure (160 mmHg) as well as hypoxic conditions (15 mmHg) for 24 hours. Cell viability, function, hypoxia-induced changes in gene expression, and cytokine secretion were then assessed. Notably, hypoxia appeared to induce a decrease in viability and increasing islet density exacerbated the observed increase in cellular apoptosis as well as the loss of function. These changes were also associated with an increase in inflammatory gene transcription. Taken together, these data indicate that when a high number of islets are confined to a small space under hypoxia, cell viability and function are significantly impacted. Thus, in order to improve islet survival in this environment during transplantation, oxygenation is of critical importance.

## INTRODUCTION

Islet transplantation is a minimally invasive therapy recommended for patients with brittle diabetes. Over the last fifteen years, the outcome of this therapy has improved, with a 3 year graft survival rate of 44% [1] as well as a decreased insulin requirement even after partial loss of the graft [2-6]. However, the main advantage of this procedure for diabetic patients is that even if they must be put back on insulin, there is a residual protective effect from hypoglycaemic unawareness episodes thanks to a basal level of C-peptide ( $<0.3$  g/L) [1]. Although this treatment option appears to have a number of positive consequences, the negative effects and limitations of the transplant, including the pancreatic requirements, immune suppressive regimen, low rate of insulin independence, short lifespan of the graft, and the recurrence of autoimmunity, have restricted the use of this therapy, particularly when treating children [6,7].

To address these issues, bioartificial pancreases that encapsulate the cells in a selective shield which lets nutrients and insulin cross the barrier while stopping immune cells and effectively hiding the graft from the host are currently being developed [2,6]. To immune-isolate the islet cells in this manner, one of two strategies is typically utilized, microencapsulation or macroencapsulation. For the first, one to four islets are encapsulated in a microsphere of polymer [2]. This polymer facilitates the exchange of oxygen and nutrients, but makes it difficult to retrieve the graft post-transplantation. In contrast, for macroencapsulation, islets are gathered in one or a few distinct devices (e.g., a sheet of polymer, cylindrical device, perfusion device, macrobeads or selective membrane [2-6,8]), which allow the grafted cells to be easily removed, providing an additional advantage in the favour of macroencapsulation from a regulatory point of view for clinical application [9,10]. However, the gathering of these cells can be detrimental as a lower level of oxygen is available in the device and the islets are confined to a much more limited area, potentially increasing the local consumption of oxygen and amplifying the hypoxic conditions surrounding the cells [6].

Physiologically, islets in a normal pancreas make up less than 2% of the whole organ, but use approximately 10% of the total oxygen supply. These cells are highly vascularized and not physiologically prepared to face hypoxia. During islet transplantation in the liver, an oxygen partial pressure ( $PO_2$ ) of 5–10 mmHg has been shown to be enough for their survival [11], but this likely only reflects the level necessary when the islets are directly connected to the vascular system. In a bioartificial pancreas, islets cannot be vascularized as this will disrupt the immune-isolation characteristics necessary for their function. Thus, in these devices, oxygen can only be accessed via membrane diffusion, and the  $PO_2$  in a macroencapsulation device is around 15 mmHg (subcutaneously or intraperitoneally [12-15]). It is unlikely that this level is high enough for the cells to function efficiently.

The consequences of hypoxia on islets, including impaired survival and function [16,17] and increased inflammation [18] associated with the recruitment and activation of host macrophages and leukocytes in the implantation site [19], are well established. Inflammatory molecules such as cytokines and chemokines promote insulinitis and  $\beta$  cell destruction together with an increased production of reactive oxygen species (ROS). The main strategy used to counteract hypoxia-induced loss of islets is to overload the devices with cells with the hope that a few will survive and help reverse the effects of diabetes. In fact, experiments performed with the TheraCyte<sup>®</sup> device, which uses 1,000 islets per device, have demonstrated a 6 month survival period for the graft in an immunized rat [20]. Unfortunately, this previous study only focused on islet function 1 month after transplantation. Furthermore, no data are available

concerning islet oxygenation, and the authors have only assumed that the vascularisation surrounding the device was able to bring enough oxygen for islet survival. Notably, one of the primary concerns with increasing the number of islets in the confined environment of the device, is that even more oxygen will be consumed [21,22], further exacerbating the localized hypoxia.

The number of islets transplanted is also depending on the size of the patient. For instance, a patient that weighs 50 kg would require a graft of 500,000 Islet Equivalent (IEQ). In order to maintain a sufficient number of functioning islet cells, the typical preconized culture density is 150 Islet Equivalent (IEQ)/cm<sup>2</sup> [21], which means that to transplant this small patient, a single 3,333 cm<sup>2</sup> device or 500 small 6.67 cm<sup>2</sup> devices would need to be inserted, which is impossible. Thus, it is essential to increase the number of survival cell/cm<sup>2</sup>.

In the present study, we investigated the effects of islet density in a cellular context that mimics that found in smaller sized bioartificial pancreases using under 15 mmHg of oxygen. In doing so, we sought to establish the effects of hypoxic islet confinement on islet survival, function, and inflammatory potency in order to develop new strategies to improve the outcome of islet transplantation in patients with diabetes.

## **MATERIALS AND METHODS**

### *- Islet cell isolation and culture:*

#### Animals

Male Wistar rats were supplied by Janvier laboratory (Le Genes St Isle, France). All rats were housed in pathogen-free conditions, in standard collective cages, in a temperature-controlled room ( $23 \pm 1^\circ\text{C}$ ) with a 12-h light/12-h darkness cycle. They were fed SAFE-A04 (Villemoisson-sur-Orge, France) food and water *ad libitum*. All experiments were performed according to the National Institutes of Health and local ethical committee (CREMEAS) guidelines (authorization number: C67-482-28).

#### Islet isolation

Pancreatic islets were isolated from adult Wistar rat pancreas (weight 200–250 g) using standard collagenase (Sigma-Aldrich, St. Louis, MO, USA) digestion and Ficoll (Eurobio, Les Ulis, France) purification. Islets were cultured in Medium 199 containing 5.5 mM glucose (Gibco, Life Technologies, Paisley, Scotland) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), and 1% antibiotic/antimycotic (ABAM; 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of Fungizone; Gibco). After culturing for 24 hours in 25 cm<sup>2</sup> flasks (Cellstar, Greiner Bio-One GmbH, Frickenhausen, Germany), three different islet densities (150, 300, and 600 IEQ/cm<sup>2</sup>) were seeded in 48-well plates (Greiner). The 150 IEQ/cm<sup>2</sup> density was considered as the control condition in these experiments. Islets were cultured at the specified density for 24 hours in a humidified incubator (StemCell Technologies, Canada) at 37°C under normal atmosphere (PO<sub>2</sub>: 160 mmHg) as well as hypoxia conditions (PO<sub>2</sub>: 15 mmHg).

*- Oxygen measurements:*

Oxygen measurements were carried out with a four-channel fiber-optic oxygen meter (Oxy-4) and non-invasive oxygen sensors (Presens, Germany). Briefly, oxygen acts as a dynamic fluorescence quencher of a luminophore in a polymer matrix. Spots, made of this polymer matrix, were glued onto the bottom of each culture well (Presens, Germany), allowing non-invasive measurements of oxygen to be taken through the plastic from the outside. An optic fibre connected to the OXY 4 oxygen meter was guided and positioned from the outside of the chamber to the spot. The data are expressed in mmHg and were taken at time 0 (t<sub>0</sub>) before placing the plates into the incubators as well as after the 24 h incubation period.

*- Islet viability:*

Viability of 10 islets from each condition were analysed by fluorescein diacetate/propidium iodide staining (FDA/PI, Sigma) by three independent investigators. The ratio of green to red cells provided the percentage of islet viability. Images were obtained on a Nikon Eclipse 50i microscope with Nis-Element-BR software (Nikon, Amstelveen, Netherlands).

*- ATP levels:*

A total of 50 islets from each condition were handpicked and ATP was extracted with mammalian cell lysis solution. Levels of ATP were assessed using an ATPlite kit (PerkinElmer, Inc., Waltham, MA, USA) following the manufacturer's instructions. Results are expressed as  $\mu\text{M}/50$  islets.

*- Islet functionality:*

A subset of islets (n = 10) from each experimental condition were washed extensively and incubated in Krebs Ringer bicarbonate (KRB) solution with 10% FBS and 4.4 mmol/L of glucose (Sigma). Islets were then stimulated with KRB solution containing 10% FBS and 22.6 mmol/L of glucose. Each incubation step was performed for 90 min at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Supernatants were then collected and stored at -80°C. Insulin measurements were performed using a rat insulin enzyme linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden). Results are expressed as a stimulated index (SI) defined as the ratio of stimulated versus basal insulin secretion.

*- Real-Time PCR:*

Total cellular RNA was extracted from approximately 400 islets from each condition using a Qiagen RNeasy Mini Kit (Qiagen, CA, USA), following the manufacturer's instructions. Extracted RNA was reverse transcribed with a RT<sup>2</sup> First Strand Kit (Qiagen), according to the manufacturer's instructions. The resulting cDNAs were diluted 1/100 in RNase free water. Real-Time PCR reactions were performed on a MyiQ Real-Time PCR System (Qiagen) using a QuantiTect SYBR Green PCR kit (Qiagen). Mouse 60S acidic ribosomal protein P1 (Rplp1), mouse peptidylprolyl isomerase A (Ppia), and mouse RNA ribosomal 1 (Rnr1) were chosen as the housekeeping genes (Table 1), and data were analyzed using the  $\Delta\Delta\text{Ct}$  method. The primers for hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ), prostaglandin-endoperoxide synthase 2 (Ptgs2/COX-2), and heme oxygenase 1 (Hmox1/HO-



1) used in this study were all purchased from Qiagen and are listed in Table 1. Amplification was run using a maximum of 35 cycles.

*- Protein extraction:*

The total protein content was extracted from 300 islets from each condition using M-PER Mammalian Protein Extraction Reagents and HALT™ Protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Illkirch-Graffenstaden, France). Protein concentration was determined using the Bradford micro-method (Bio-Rad, Life Science Group, Marnes-la-Coquette, France) and are expressed as mg/mL.

Caspase 3 activation in these cytosolic protein extracts of the islets was assessed with a Quantikine® ELISA kit (R&D Systems®, Minneapolis, MN, USA). Results are expressed in ng/mg of protein.

*- HIF-1  $\alpha$  activation (ELISA):*

HIF-1 $\alpha$  activation was quantified by TransAM™ HIF-1 (Active Motif, Belgium) following the manufacturer's instructions. 20 $\mu$ g of the total protein extraction were used to assess HIF-1 $\alpha$ . HIF dimers bind specifically to hypoxia response element (HRE) immobilized in the 96-well plate. HIF dimers are detected by HIF-1 $\alpha$  antibody (dilution: 1/500) and a secondary antibody conjugated to HRP (dilution: 1/1000). The results are expressed in OD.

*- Inflammation:*

An aliquot of the culture medium was used to measure the concentration of secreted interleukin (IL)-6 (R&D Systems®, Minneapolis, MN, USA) under each condition using an ELISA kit. Results are expressed in pg/mL/IEQ.

*- Histology:*

A total of 300 islets from each condition were harvested, washed, and snap-frozen in optimal cutting temperature compound (Tissue O.C.T. Labonord, Templemars, France), then sliced in 12- $\mu$ m sections. Apoptotic cells were stained using a Terminal deoxynucleotidyl transferase dUTP Nick End Labelling (TUNEL) assay (Millipore, Molsheim, France) and observed by fluorescence microscopy.

Hypoxic staining was performed using a Hypoxyprobe™-1 Kit (HPI, Inc. Burlington, MA, USA). Solid pimonidazole HCl was added in the islet cell cultures (150  $\mu$ M). After 24h culture, islets were fixed with 2% paraformaldehyde (PFA) for 10 min, and then incubated with primary and secondary antibodies. The antibodies used in this study include anti-mouse immunoglobulin (IgG<sub>1</sub>, 1:50 dilution; HPI, Inc.) and Alexa Fluor 488 goat anti-mouse IgG (dilution 1:200; Life technologies, Carlsbad, CA, USA).

*- Statistical analysis:*

Statistical analyses were performed using Statistics software (StatSoft, Maisons-Alfort, France). Results were analysed with one-way analysis of variance (ANOVA) followed by post-hoc Tukey honest significant difference (HSD) testing for the parametric data or Kruskal-Wallis for the non-parametric analysis. Results are expressed as mean  $\pm$  SEM. A p value less than 0.05 was considered statistically significant.

## RESULTS

### *Oxygen measurement*

At  $t_0$ , the  $PO_2$  was found to be relatively similar for all three culture densities (150, 300, and 600 IEQ/cm<sup>2</sup>) under both normal and hypoxic conditions (Table 2). However, after 24 hours of exposure to hypoxia ( $PO_2$ : 15 mmHg), the  $PO_2$  of the control islet cultures (150 IEQ/cm<sup>2</sup>) dropped to  $14.3 \pm 6.2$  mmHg compared to  $121 \pm 9.7$  mmHg under normal conditions. We also observed a decrease in the  $PO_2$  in the cells cultured at the 300 and 600 IEQ/cm<sup>2</sup> densities ( $99.5 \pm 12.7$  mmHg and  $80.5 \pm 8.7$  mmHg, respectively) compared to the control after being in culture for 24 hours under normoxic conditions. Furthermore, the hypoxia-induced drop in the  $PO_2$  observed in the control islets was exacerbated when the cell density was increased to 300 and 600 IEQ/cm<sup>2</sup> ( $10.5 \pm 5.1$  mmHg and  $7.6 \pm 3.2$  mmHg, respectively).

### *Islet viability*

Under hypoxic conditions, the viability of the islets cultured for 24 hours at a density of 150 IEQ/cm<sup>2</sup> was significantly decreased compared to those under normal atmosphere (Figure 1A). A similar trend was observed at the higher densities, with the greatest difference being observed at 600 IEQ/cm<sup>2</sup>. Representative FDA/PI stained images of the normal and hypoxic cells at each density are shown in Figure 1B. These data are supported by a significant increase in Caspase 3 protein expression (Figure 1C;  $p < 0.05$ ) as well as an observed increase in TUNEL staining (Figure 1D) in the hypoxia treated cells compared to the normoxia treated cells. Taken together, these data indicate that islet density/confinement did not have a significant effect on the percentage of viable cells when cultured in normoxic conditions, but does appear to intensify the effects of hypoxia.

### *Hypoxia marker expression*

Not surprisingly, HIF-1 $\alpha$  mRNA expression (Figure 2A) and hypoxiprobe staining (Figure 2C) were significantly increased in the control islets cultured under hypoxic conditions compared to those cultured under normoxic conditions. Furthermore, the density/confinement of the islets also appeared to have a significant impact on the HIF-1 $\alpha$  mRNA expression and hypoxiprobe staining when the cells were cultured under hypoxic conditions. A correlation between the increase in density and the activation of HIF-1 $\alpha$  protein (Figure 2B) appeared under hypoxia and became significant for 600 IEQ/cm<sup>2</sup> ( $p < 0.05$ ) compared to normoxic conditions. In fact, the levels of these hypoxia markers appeared to increase with the increasing level of hypoxia, indicating a particularly significant increase in the cells cultured at 600 IEQ/cm<sup>2</sup> ( $p < 0.05$ ) compared to the cell cultured at 150 IEQ/cm<sup>2</sup> under hypoxic conditions.

### *Islet functionality*

Islet function, in terms of ATP levels in the cell, was also evaluated. These data demonstrate that, compared to normoxia, hypoxia induced a significant decrease in the ATP content regardless of cell culture density ( $p < 0.05$ ). However, this decrease in ATP content was significantly more prominent for the cells culture in the more confined densities compared to those cultured at 150 IEQ/cm<sup>2</sup> ( $p < 0.05$ ) (Figure 3A). These results were also supported during our analysis of insulin secretion. We observed a significant loss of function

when the cells were cultured under hypoxia compared to normoxia ( $p < 0.05$ ). At 300 IEQ/cm<sup>2</sup> and 600 IEQ/cm<sup>2</sup>, under normoxic conditions we observed a slight decrease in the stimulation index for both densities compared to the control density; however, these islets were still considered functional as their stimulation indices were around or over 2. Under hypoxia, confinement tended to emphasize the loss of function observed in the control density, with the indices being significantly reduced to less than 1 for both the 300 IEQ/cm<sup>2</sup> and 600 IEQ/cm<sup>2</sup> densities compared to 150 IEQ/cm<sup>2</sup> (Figure 3B).

### *Inflammation*

In order to evaluate the effects of cell density on the inflammatory response induced during hypoxia, we measure the relative mRNA expression of two inflammation markers, COX-2 and HO-1, as well as the secretion of IL-6. We observed that hypoxia induced a significant upregulation of both COX-2 (Figure 4A,  $p < 0.05$ ) and HO-1 (Figure 4B,  $p < 0.05$ ) mRNA expression compared to normoxia, and these increases in gene expression appear to occur independently of islet density. Interestingly, this response appears to be significantly greater in the control cells compared to the higher densities. Furthermore, IL-6 secretion did not appear to be influenced by the change in PO<sub>2</sub> to hypoxic levels at any cell density. However, IL-6 secretion is seemingly controlled independently of PO<sub>2</sub>, as a significant increase was observed in for both of the higher densities under normoxic conditions compared to the control cells as well as under hypoxic condition for the 300 IEQ/cm<sup>2</sup> density compared to the control ( $p < 0.05$ ) (Figure 4C).

## **DISCUSSION**

In the present study, we have shown that the combination of hypoxia and increased islet density/confinement, which are the typical conditions in a bioartificial pancreas, cause an increased level of islet cell death. Indeed, when the recommended culture density was doubled (300 IEQ/cm<sup>2</sup>) or multiplied by four (600 IEQ/cm<sup>2</sup>) and maintained under hypoxic conditions, the overall survival of the islets was very poor, with an observed activation of apoptosis and necrosis factors as well as loss of function, and pro-inflammatory cytokine secretion. To our knowledge, this is the first published study demonstrating the specific effects of hypoxia on confined islet cell survival and function.

In order to effectively and efficiently combat diabetes, it is essential that bioartificial pancreases maintain a sufficient number of viable, fully functioning islets cells. Based on clinical hepatic islet transplantations, 10,000 IEQ/kg are needed to reach normoglycaemia [1]. Unfortunately, it has been estimated that approximately 40% of the transplanted islets will die because during the instant blood mediated inflammatory reaction (IBMIR) [23], and another 10 to 20% will die during vascularization [24]. These losses can be avoided when using a bioartificial pancreas. However, in contrast with liver transplantation, islets will never be vascularized in a device due to the immune-isolation required. Therefore, no oxygen or nutrients will be delivered directly to islets by the vessels, because only the device can be vascularized [3,25]. Oxygen will be delivered from the surrounding vessels to the device, or via diffusion in the case of non-vascularized devices, and will then need to diffuse into the cells, resulting in a low cellular PO<sub>2</sub> (around 15 mmHg in the case of non-vascular devices) [15].

Notably, in an alternative study, a  $\beta$ -air II device, with a  $PO_2$  between 304–198 mmHg in the gas chamber and more than 2,000 IEQ/cm<sup>2</sup>, was used once a day to increase oxygenation of encapsulated islets in rats [26]. However, a previous report suggests that such a fluctuation in oxygen, going from hyperoxia to hypoxia, may cause oxygen toxicity [27]. Moreover, the device is not completely independent from external intervention, and it is possible that the oxygen injected into the chamber can actually generate ROS [28]. It is likely that the complications caused by such oxygenating devices will prevent their widespread clinical use.

Thus, in order to compensate for islet cell death in bioartificial pancreases, a higher number of cells are typically cultured in the device. Our results in the present study show that when the number of islets was doubled or increased four times the recommended density for islet culture, the oxygen availability decreased. In fact, the oxygen concentration dropped from 15 mmHg to 7 mmHg at the highest cell density, a change that will undoubtedly have repercussions in terms of islet survival. Interestingly, it has been shown that the cells consuming oxygen will actually create an oxygen gradient [21] further depriving other cells from oxygen. Moreover, under hypoxic conditions, we observed the effects of cell density to be amplified, leading to cell death and loss of function in less than 24 h. Zheng *et al.* [29] previously demonstrated that adaptation to hypoxia is difficult for normal, untreated islets, suggesting that it could be even more difficult for transplanted/cultured islets. To this end, we have shown that hypoxic conditions combined with confinement triggered HIF-1 $\alpha$  transcription, which was also correlated with activation of COX-2 (involved in inflammatory process) and HO-1 (involved in oxidative stress) mRNA expression [19]. These data imply that the cells are at least attempting to activate their endogenous defense system against the lack of oxygen. Notably, the increased expression of both COX-2 and HO-1 in the higher density cells was significantly lower than the increase observed in the cells cultured at a normal density and appears to be insufficient, leading to an increased rate of apoptosis in the more confined cells. We also observed that secretion of IL-6 is increased as the level of cell confinement increased, regardless of oxygen availability. The role of IL-6 during islet cell survival is controversial in the literature. IL-6 is secreted as a response to cell stress and some studies have suggested that it is deleterious for islet cells [19], while others indicate that it is beneficial for their function [30]. Here, IL-6 secretion appears to be related to confinement only. However, it is possible that the 24 hour time point used in this study may have been too early to see the effects of hypoxia on IL-6 secretion. The observed increase in IL-6 secretion in the more confined cell cultures could also potentially be attributed to the paracrine and autocrine effects this cytokine has on its own secretion. Thus, the more cells there are in a limited space, the more IL-6 will be secreted as each islet will be more able to induce the surrounding cells. As this cytokine is known to play a significant role in inflammation [31], its secretion in the more confined cells could affect the recruitment and activation of host macrophages and leukocytes around the device, potentially increasing fibrosis and preventing good vascularization.

In terms of islet cell function, the decrease we observed in cellular ATP was enhanced under hypoxia, a phenomenon that is likely due to ATP leakage, which has been described in cases of organ ischemia [32-34]. Under low  $PO_2$ , the tricarboxylic acid cycle cannot be used and the switch for anaerobic respiration is triggered, but this alternative pathway produces a much lower amount of ATP. It was, therefore, not surprising that the level of ATP was significantly decreased during hypoxia. This loss of ATP was also correlated with a decrease in the cell's ability to respond to glucose stimulation. Notably, ATP and insulin are strongly correlated, and the conversion of proinsulin to insulin and C-peptide is, at least in part, stimulated by

ATP-dependent processes [35]. This relationship likely plays a significant role in the decrease function observed in the cells cultured at a higher density under hypoxic conditions.

## **CONCLUSION**

We have shown that islets in a confined environment and under hypoxia suffer from hypoxia-induced apoptosis, oxidative stress, and inflammation, and are nonfunctional after only 24 hours of incubation. In order to improve islet survival in a bioartificial pancreas, the effects of hypoxia need to be reduced, as well as the inflammatory response, in order to improve islet survival and function after transplantation. While this study highlights the need for these changes, additional work is necessary in order to develop new strategies to improve the outcome of islet transplantation in patients with diabetes.

## **NOMENCLATURE**

PO<sub>2</sub> – oxygen partial pressure, ATP – adenosine triphosphate, Rplp1 – ribosomal protein P1, Ppia – peptidylprolyl isomerase A, Rnr1 – RNA ribosomal 1, Hif-1 $\alpha$  – hypoxia-inducible factor 1 alpha, Ptg2/Cox-2 – prostaglandin-endoperoxide synthase 2, Hmox1/Ho-1 – heme oxygenase 1, ROS – reactive oxygen species, IEQ – islet equivalent

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## **DUALITY OF INTEREST**

The authors declare that there is no duality of interest associated with this manuscript.

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## Table Legends

### Table 1: qPCR Primers

**Table 2.** Oxygen measurements at time 0 ( $t = 0$ ) and after 24 hours ( $t = 24$  h) for each islet culture density (150, 300, and 600 IEQ/cm<sup>2</sup>) under normoxic (PO<sub>2</sub> 160 mmHg) and hypoxic (PO<sub>2</sub> 15 mmHg) conditions The difference between T0 and T24 is represented by the p values. The difference between densities within the same condition is represented by \*.

## Figure legends

**Fig. 1.** Islet viability. (A) Islet viability at each cell density after 24 hours in culture under both normoxic (PO<sub>2</sub> 160 mmHg) and hypoxic (PO<sub>2</sub> 15 mmHg) conditions. (B) Representative fluorescein diacetate/propidium iodide (FDA/PI) stained images of islets cultured at 150, 300, or 600 IEQ/cm<sup>2</sup> under normoxia and hypoxia. Green staining (FDA) indicates the cells are alive, while red staining (PI) indicates that the cells are dead. Scale bars = 100 μm. (C) Islet apoptosis at each cell density after 24 hours in culture under both normoxic (PO<sub>2</sub> 160 mmHg) and hypoxic (PO<sub>2</sub> 15 mmHg) conditions. (D) Representative images of TUNEL stained cells cultured at 150, 300, or 600 IEQ/cm<sup>2</sup> under normoxia and hypoxia. Blue staining highlights the cell nuclei (DAPI), and the green staining indicates apoptosis. Scale bars = 50 μm. (E) Caspase-3 activation at each cell density after 24 hours in culture under both normoxic (PO<sub>2</sub> 160 mmHg) and hypoxic (PO<sub>2</sub> 15 mmHg) conditions. <sup>a</sup>p < 0.05 compared to the control, <sup>aaa</sup>p < 0.001 compared to 600 IEQ/cm<sup>2</sup> normoxia.

**Fig. 2.** Hypoxia marker expression. (A) Levels of hypoxia-inducible factor 1 alpha (HIF-1α) mRNA expression after 24 hours in culture under normoxia (PO<sub>2</sub> 160 mmHg) and hypoxia (PO<sub>2</sub> 15 mmHg) at 150, 300, and 600 IEQ/cm<sup>2</sup>. (B) Translocation and activation of HIF-1 α protein was performed after 24 hours in each density (150, 300, and 600 IEQ/cm<sup>2</sup>) under normoxia and hypoxia conditions. (C) Representative images of hypoxiprobe stained cells at 150, 300, and 600 IEQ/cm<sup>2</sup> under normoxia and hypoxia. Blue staining highlights the cell nuclei (DAPI), and the green staining indicates piminidazole (hypoxia marker) expression. Scale bars = 50 μm. <sup>aaa</sup>p < 0.001 compared to the same concentration under normoxic conditions, <sup>&</sup>p < 0.05 compared to 150 IEQ/cm<sup>2</sup> under hypoxic conditions, <sup>a</sup>p < 0.05 compared to the control.

**Fig. 3.** Islet functionality. (A) ATP levels were measured after 24 hours of culture at the 150, 300, and 600 IEQ/cm<sup>2</sup> under normoxic (PO<sub>2</sub> 160 mmHg) and hypoxic (PO<sub>2</sub> 15 mmHg) conditions. (B) Insulin response to glucose stimulation was measured after 24 hours in culture using a stimulation index. Each density (150, 300, and 600 IEQ/cm<sup>2</sup>) was evaluated under both normoxic (PO<sub>2</sub> 160 mmHg) and hypoxic (PO<sub>2</sub> 15 mmHg) conditions. <sup>a</sup>p < 0.05 compared to the control, <sup>#</sup>p < 0.05 compared to 150 IEQ/cm<sup>2</sup> under normoxia, <sup>&</sup>p < 0.05 compared to 150 IEQ/cm<sup>2</sup> under hypoxia.

**Fig 4.** Inflammation (A) Levels of Cox-2 mRNA expression in islets after 24 hours in culture at 150, 300, and 600 IEQ/cm<sup>2</sup> under normoxic (PO<sub>2</sub> 160 mmHg) and hypoxic (PO<sub>2</sub> 15 mmHg) conditions. (B) Levels of Ho-1 mRNA expression after 24 hours in culture under the specified conditions. (C) IL-6 secretion was measured in islet medium after 24 hours in culture under the specified conditions. <sup>a</sup>p < 0.05 compared to the control, <sup>aaa</sup>p < 0.001 compared to 150 IEQ/cm<sup>2</sup> under normoxic conditions, <sup>#</sup>p < 0.05 compared to 150 IEQ/cm<sup>2</sup> under normoxic conditions, <sup>&</sup>p < 0.05 and <sup>&&&</sup>p < 0.001 compared to 150 IEQ/cm<sup>2</sup> under hypoxic conditions.



**Table**

**Table 1**

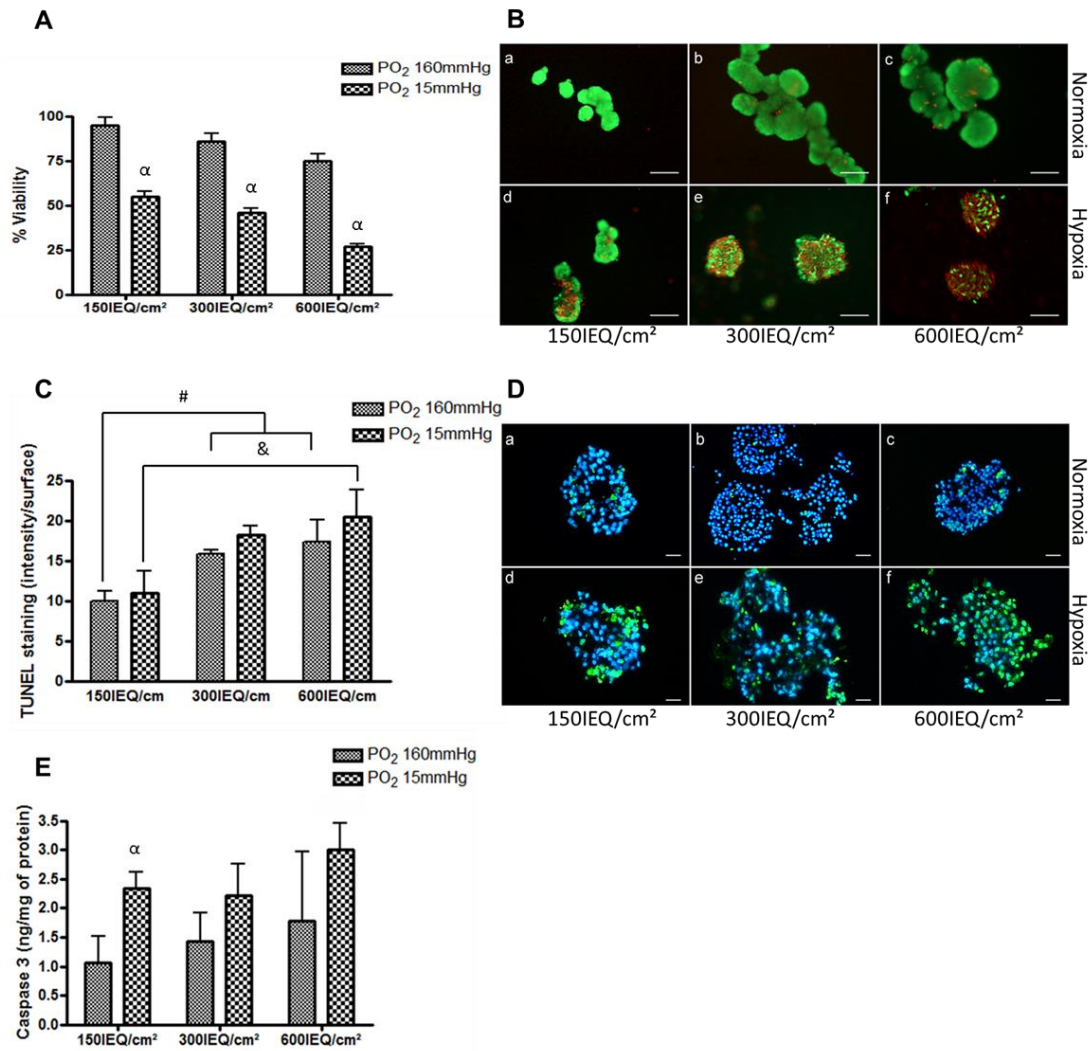
<b>Primers</b>	<b>Reference</b>
<b>Rn_Rnr1_1_SG QuantiTect primer assay</b>	<b>QT00199374</b>
<b>Rn_Ppia_1_SG QuantiTect primer assay</b>	<b>QT00177394</b>
<b>Rn_Rplp1_2_SG QuantiTect primer</b>	<b>QT01745625</b>
<b>Rn_Hif1a_1_SG QuantiTect primer assay</b>	<b>QT00182532</b>
<b>Rn_Ptgs2_1_SG QuantiTect primer assay</b>	<b>QT00192934</b>
<b>Rn_Hmox1_1_SG QuantiTect Primer Assay</b>	<b>QT00175994</b>

**Table 2**

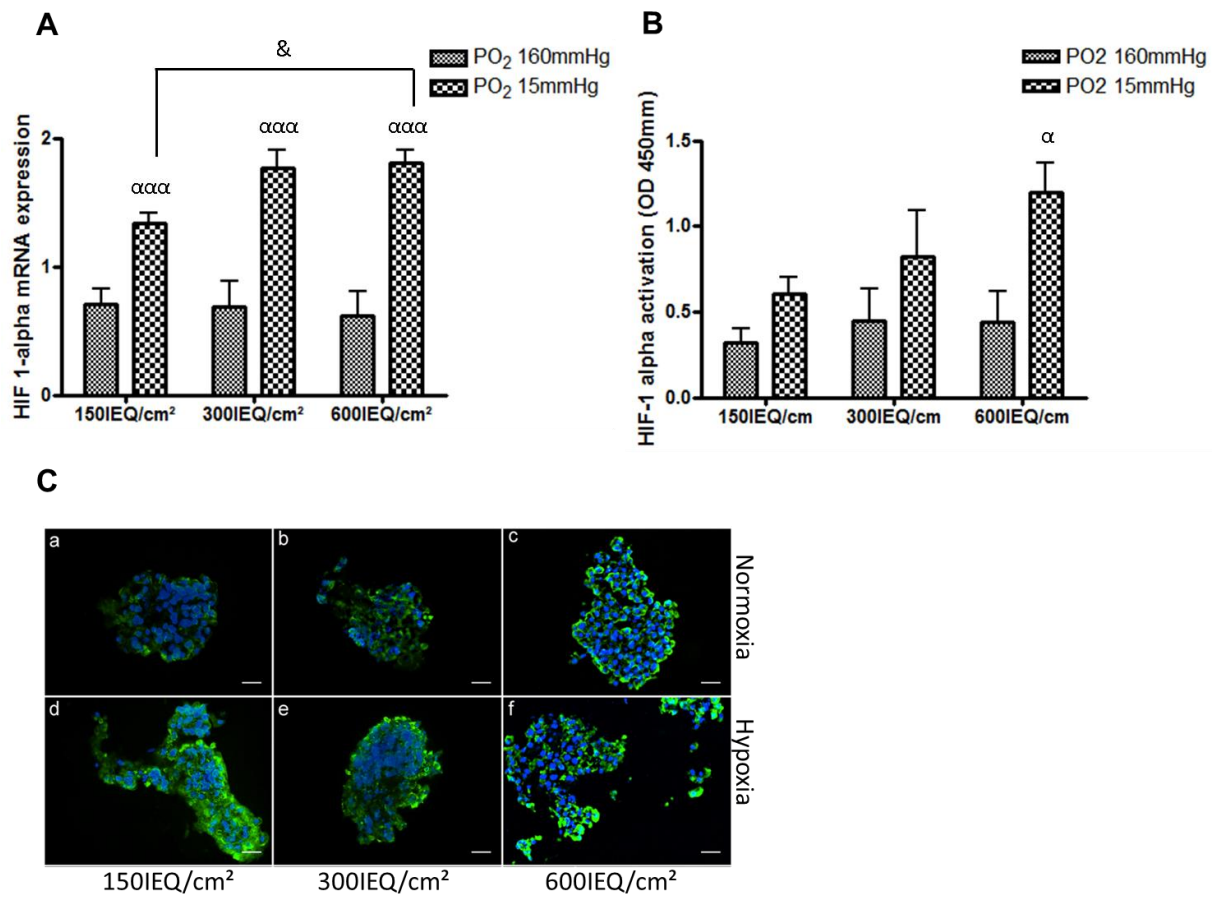
	<i>PO<sub>2</sub> 160mmHg</i>			<i>PO<sub>2</sub> 15mmHg</i>		
	<b>150IEQ/cm<sup>2</sup></b>	<b>300IEQ/cm<sup>2</sup></b>	<b>600IEQ/cm<sup>2</sup></b>	<b>150IEQ/cm<sup>2</sup></b>	<b>300IEQ/cm<sup>2</sup></b>	<b>600IEQ/cm<sup>2</sup></b>
<b>t=0</b>	<b>143.8±3.8</b>	<b>143.1±4.7</b>	<b>133.9±5.1</b>	<b>150.1±3.2</b>	<b>142.4±3.3 *</b>	<b>133.0±4.6*</b>
<b>t=24h</b>	<b>121.6±9.7</b>	<b>99.5±12.7*</b>	<b>80.5±8.7***</b>	<b>14.3±6.2</b>	<b>10.5±5.1</b>	<b>7.6±3.2</b>
<b>p Value</b>	<b>0.03</b>	<b>0.00098</b>	<b>0.00007</b>	<b>0.034</b>	<b>0.000010</b>	<b>0.00000</b>

# Figures

## Fig 1



**Fig 2**



**Fig 3**

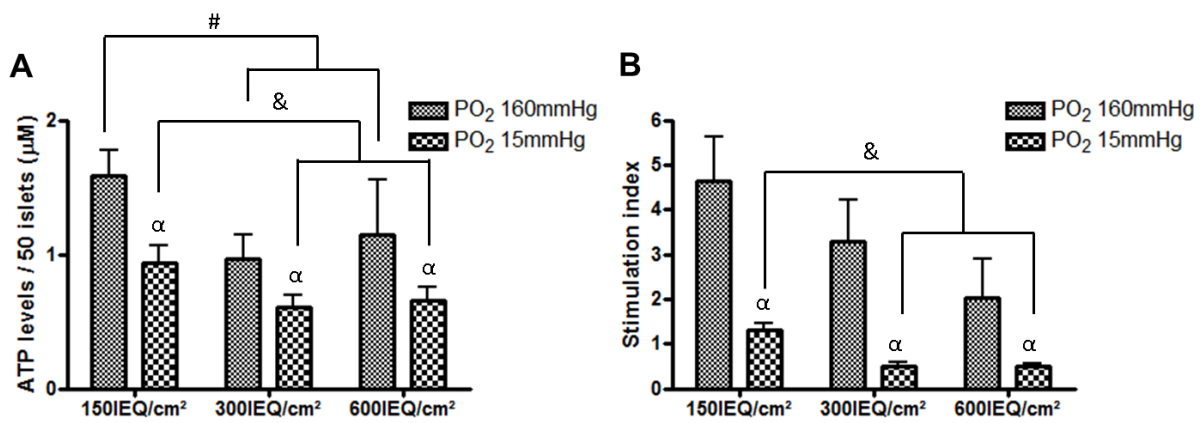
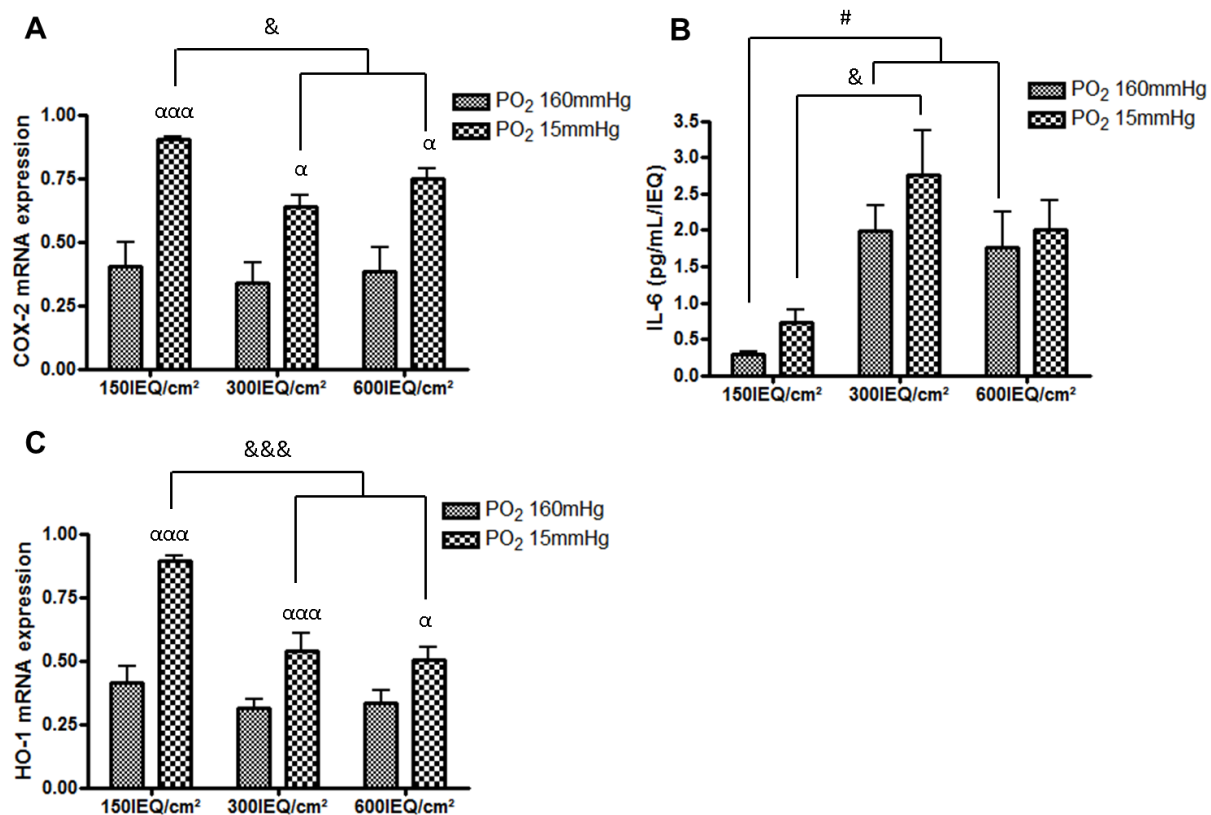


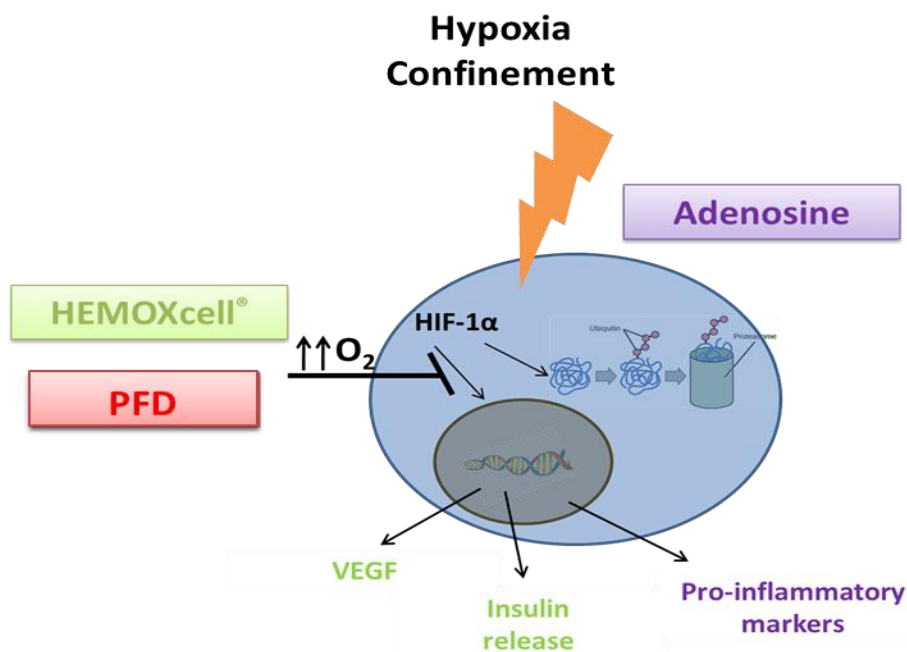
Fig 4



## 4.2 Improvement of hypoxia in encapsulated islet

In the previous work we have shown that hypoxia is one of the major problems in encapsulated islets, which is worsen by confinement, the main characteristics in the bioartificial pancreas. In the second part of this study we have tested different oxygen carriers as an oxygen supply in order to improve islet survival and functionality under hypoxic conditions.

In order to reduce hypoxia levels in a confined islet density (600IEQ/cm<sup>2</sup>) different oxygen carriers like perfluorodecalin (PFD) and hemoglobin from *Arenicola marina* (HEMOXCell<sup>®</sup>) w/o adenosine (ADE) were tested *in vitro* in 600IEQ/cm<sup>2</sup> under hypoxia during 24 hours. Administration of these molecules loaded in oxygen, produced a decrease in HIF-1 $\alpha$  mRNA and protein levels improving islet oxygenation. However, VEGF release was not correlated with hypoxia due to the 24 hour time which was not enough for VEGF secretion. In contrast, hemoglobin has the capacity to upregulate VEGF release and we have shown an increase in VEGF in addition of HEMOXCell<sup>®</sup> w/o adenosine after 24 hours. In order to see how the hypoxia decrease could affect inflammation, we have studied also different markers related with pro-inflammation pathway. The addition of oxygen carriers in combination with adenosine showed an increase in the pro-inflammatory markers that can be explained by the pro-inflammatory characteristics of adenosine when specific adenosine receptors are activated. Addition of ADE, HEMOXCell<sup>®</sup> w/o adenosine and PFD+ADE, seems to increase the levels of pNF- $\kappa$ B/NF- $\kappa$ B activating the inflammation pathway. We have studied also COX-2 protein levels and patently we had the same pattern than pNF- $\kappa$ B/NF- $\kappa$ B with a higher increase in COX-2 protein levels with the addition of ADE and PFD+ADE than HEMOXCell<sup>®</sup> w/o adenosine. The quantification of IL-6 release showed an increase with the addition of ADE and PFD+ADE. The decrease in hypoxia from HEMOXCell<sup>®</sup> w/o adenosine was responsible of the restoration of islet function in a confined environment. However, ADE and PFD w/o adenosine, even if there was a decrease in hypoxia, there was not an improvement in islet functionality (**Fig. 4-2**).



**Figure 4-2** Impact oxygen carrier molecules tested in islets

The results obtained in this work are submitted in Tissue Engineering journal. **A. Rodriguez-Brotons**, W. Bietiger, C. Peronet, J Magisson, C. Mura, C. Sookhareea, N. Jeandidier, V. Polard, F. Zal, M. Pinget, S. Sigrist, E. Maillard. Comparison between perfluorodecalin and HEMOXCell<sup>®</sup> as oxygen carrier for islet encapsulation

## **Comparison between perfluorodecalin and HEMOXCell<sup>®</sup> as oxygen carrier for islet encapsulation**

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## ABSTRACT

Transplantation of encapsulated islet in a bioartificial pancreas is a promising alternative to free islet cell therapy to avoid immunosuppressive regimen. However, hypoxia in this isolated area is the major limiting factor, which induced a rapid islet loss. Moreover, the confined condition of islet in this restricted space triggers hypoxia. To improve oxygenation in such condition and prevent islet death, oxygen carriers such as perfluorocarbons and hemoglobin could have a beneficial impact. Both oxygen carriers showed beneficial effects on cells under normoxia conditions, however efficiency has never been checked in hypoxic and confined conditions. The aim of this work was to compare the effect of perfluorodecalin (PFD) and HEMOXCell<sup>®</sup>, marine oxygen carrier having properties of high oxygen sensitivity, in combination with adenosine on islet survival, on hypoxia and inflammation markers and on function, under the specific conditions of encapsulation which are hypoxia (oxygen partial pressure PO<sub>2</sub> 15mmHg) and confinement. Islets were cultured in the presence of 10% of PFD or 50µg/ml of HEMOXCell<sup>®</sup> (both with or without adenosine 1mM) for 24hours under PO<sub>2</sub> 15mmHg, and survival and function were assessed. PFD and adenosine increased cell viability, decreased hypoxia markers (HIF mRNA and protein) and had no effect on VEGF secretion. However, an increase in inflammation markers by addition of PFD and adenosine was correlated with their inability to restore islet function. On the opposite, HEMOXCell<sup>®</sup> decreased hypoxia markers and inflammation which was related to a restoration of islet function.

To conclude, it appeared that HEMOXCell<sup>®</sup>, in contrast with PFD, is able to maintain islets viability and function under drastic conditions without any impact of adenosine supplementation. The use of HEMOXCell<sup>®</sup> to improve islet survival in an encapsulation device could be an interesting strategy.



## INTRODUCTION

Cell therapy is an appealing alternative to whole pancreas transplantation for restoring physiological control of glycemia and insulin delivery in type 1 diabetic patients; however, it can only be used in patients with brittle diabetes, hypoglycemia unawareness, and poor quality of life, because the immunosuppressive regimen is more deleterious than the diabetes itself [1]. Thus, the benefit-risk balance is always closely studied for each case. The major advantage of cell therapy, contrary to whole organ transplantation, is the possibility to isolate the graft from the host using an macro-encapsulation device, i.e. bioartificial pancreas [2]. Islets in the device sense information, such as the surrounding glucose, oxygen, and nutrients, through a semi permeable barrier [3], which protects the graft and prevents the entry of molecules and immune cells that promote graft rejection [4]. Islets can respond to glucose stimulation to secrete insulin, which crosses the barrier to reach the bloodstream [2].

However, the main limitation of the application of this technology is the lack of oxygen inside the devices. The measurement of oxygen availability in a macroencapsulation device is typically a partial pressure of around 20 mmHg [5], which is half of the partial pressure of an islet in the vascularized pancreas [6]. In addition, the hypoxic condition in the device is worsened by increased islet density in a restricted space, which has been shown to decrease oxygen availability in a cell density-dependent manner [7, 8]. Indeed, islets consume oxygen in their vicinity to maintain their metabolism, depriving even more oxygen in their environment. Hypoxia leads to the breakdown of ATP [9], which induces the loss of islet function and finally cell death. In a previous work, we showed that confinement in combination with hypoxia in a model that mimics the environment of bioartificial pancreas decreased the survival rate of islets. In the same study, we also showed that islets suffered from hypoxia and inflammation, leading to apoptosis [10]. Thus, usage of hemoglobin [11, 12] or perfluorocarbons (PFCs) [13, 14] could be useful in that context.

PFCs are inert compounds in which the hydrogen atoms are replaced by fluorine atoms. This unique feature gives PFCs the particular ability to dissolve and transfer molecular oxygen [15]. Because of physical dissolution rather than chemical binding, and high diffusion rates related to low intermolecular cohesion forces in liquid PFCs, the partial pressure of O<sub>2</sub> in PFC emulsions is in equilibrium with the surrounding media [16]. PFCs have been used in organ preservation and tested on islet cultures under normoxic conditions. Perflubron and perfluorodecalin are both able to prevent hypoxia from occurring in the islet core during culture at atmospheric O<sub>2</sub> partial pressure. PFCs can increase the oxygen gradient around islets, which allows oxygen to penetrate more deeply into the organoid [8, 17, 18]. Previous studies have shown that the combination of PFCs and adenosine, during the process of pancreas preservation and digestion improved the levels of ATP resulted in improved islet survival [19, 20]. Adenosine is an important substrate for ATP production, and ATP is implicated in islet metabolism including insulin secretion [21, 22], and protection against oxidative stress [22].

Hemoglobin, a natural oxygen carrier, is composed of 4 globins (2 alpha and 2 beta chains) for fixing 4 molecules of oxygen simultaneously [23]. HEMOXCell<sup>®</sup>, a marine extracellular hemoglobin, presents highly interesting characteristics. It is composed of 156 globins, thus is able to link 156 O<sub>2</sub> molecules when saturated. This molecule has been shown to improve cell viability and proliferation [24]; however, the oxygenation capacity of HEMOXCell<sup>®</sup> has never been tested on islets under hypoxia.

The aim of the present work was to compare the efficiency of two oxygen carriers, perfluorodecalin and HEMOXCell<sup>®</sup>, supplemented with or without adenosine, in overcoming hypoxia in conditions that mimic the bioartificial pancreas.

## MATERIAL AND METHODS

### *-Oxygen carriers:*

#### HEMOXCell<sup>®</sup>:

The extracellular hemoglobin M201 has been used to create the product designated as HEMOXCell<sup>®</sup> (HEMARINA SA, Morlaix, France). This molecule is extracted from the marine lugworm *Nereis virens*. The extraction process is realized under gentle agitation of frozen worms at 4°C, followed by a purification step and a conditioning process. HEMOXCell<sup>®</sup> has been developed to replace the way oxygen is traditionally supplied to cells from early stage R&D studies to process development and finally production of recombinant proteins at commercial scale.

#### Perfluorodecalin:

Perfluorodecalin (C<sub>10</sub>F<sub>18</sub>) (F2 Chemicals Ltd., Lancashire, UK) is a polycyclic perfluoroalkane. It is a derivative of decalin, with all of the hydrogen atoms replaced by fluorine atoms. Its density (1.92 g/cm<sup>3</sup>) and viscosity (2.61 cS) [25] are higher than those of water. This molecule is highly stable, biologically inert, and is easily eliminated by the body. PFD has been found to dissolve high concentrations of oxygen that is released when the partial pressure of oxygen is reduced in the surrounding environment.

### *- Islet cell isolation and culture:*

#### Animals

Male Wistar rats were supplied by Janvier laboratory (Le Genes St Isle, France). All rats were housed in pathogen-free conditions, in standard collective cages, in a temperature-controlled room (23 ± 1°C) with a 12-h light/12-h darkness cycle. They were fed with SAFE-A04 (Villemoisson-sur-Orge, France) food and water *ad libitum*. All experiments were performed according to the National Institutes of Health and local ethical committee (CREMEAS) guidelines (authorization number: C67-482-28).

#### Islet isolation

Pancreatic islets were isolated from adult Wistar rat pancreas (weight 200–250 g) using standard collagenase (Sigma-Aldrich, St. Louis, MO, USA) digestion and Ficoll (Eurobio, Les Ulis, France) purification. Islets were cultured in Medium M199 containing 5.5 mM glucose (Gibco, Life Technologies, Paisley, Scotland) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), and 1% antibiotic/antimycotic (ABAM; 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of Fungizone; Gibco). After culturing for 24 hours in 25 cm<sup>2</sup> flasks (Cellstar<sup>®</sup>, Greiner Bio-One GmbH, Frickenhausen, Germany), islets were seeded in 48-well plates (Greiner) in a confined density

(600 IsletEquivalent (IEQ)/cm<sup>2</sup>). Culture medium was supplemented with 10% of PFD (F2 Chemicals Ltd., Lancashire, UK) or HEMOXCell<sup>®</sup> (50µg/ml) (HEMARINA SA, Morlaix, France). Media were prepared and saturated 30 minutes with pure oxygen prior culture for loading the PFD. To ensure the homogeneity of the preparation the medium with HEMOXCell<sup>®</sup> was saturated as well even if at a partial pressure of 150 mmHg, HEMOXCell<sup>®</sup> is already saturated.

The 600 IEQ/cm<sup>2</sup> density under hypoxia was considered as the control condition in these experiments. According to our previous work, 600IEQ/cm<sup>2</sup> were cultured at the specified density for 24 hours in a humidified incubator (StemCell Technologies, Canada) at 37°C under hypoxia conditions (PO<sub>2</sub>: 15mmHg: 2% O<sub>2</sub>, 5% CO<sub>2</sub>, 93% N<sub>2</sub>, Lindegas, France). Oxygen measurements were carried out with a four-channel fiber-optic oxygen meter (Oxy-4) and noninvasive oxygen sensors (Presens, Germany). Spots were glued onto the bottom of each culture well (Presens, Germany), allowing noninvasive measurements of oxygen to be taken through the plastic from the outside. An optic fiber connected to the OXY 4 oxygen meter was guided and positioned from the outside of the chamber to the spot. The data are expressed in mmHg and were taken at time 0 and 24hours before and after placing the plates into the hypoxic chamber.

*- Islet viability:*

Viability of 10 islets from each condition was analyzed using LIVE/DEAD<sup>®</sup> Viability/Cytotoxicity Assay Kit (ThermoFisher Scientific, Illkirch-Graffenstaden, France) following the manufacturer's instructions. Live cells are distinguished by the polyanionic dye calcein AM which is retained within live cells producing a green fluorescence. EthD-1 enters cells with damages membranes and bind to nucleic acids producing a red fluorescence in dead cells. The ratio of green to red cells provided the percentage of islet viability. Results from sampling (n=3) are represented as mean of percentage ± SEM. Images were obtained on a Nikon Eclipse 50i microscope with Nis-Element-BR software (Nikon, Amstelveen, Netherlands).

*- Islet functionality:*

A subset of islets (n = 10) from each experimental condition were washed extensively and incubated in Krebs Ringer bicarbonate (KRB) solution with 10% FBS and 4.4 mmol/L of glucose (Sigma). Islets were then stimulated with KRB solution containing 10% FBS and 22.6 mmol/L of glucose. Each incubation step was performed for 90 min at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Supernatants were then collected and stored at -80°C. Insulin measurements were performed using a rat insulin enzyme linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden). Insulin content was normalized to the protein extraction for each condition. Insulin content was expressed as µg/gr of protein and as a stimulated index (SI) defined as the ratio of stimulated versus basal insulin secretion. Islet preparation is functional for a SI >1.

*- Real-Time PCR:*

Total cellular RNA was extracted from approximately 400 islets from each condition using a Qiagen RNeasy Mini Kit (Qiagen, CA, USA), following the manufacturer's instructions. Extracted RNA was reverse transcribed with a RT<sup>2</sup> First Strand Kit (Qiagen), according to the manufacturer's instructions. The resulting cDNAs were diluted 1/100 in

RNase free water. Real-Time PCR reactions were performed on a MyiQ Real-Time PCR System (Qiagen) using a QuantiTect SYBR Green PCR kit (Qiagen). Mouse 60S acidic ribosomal protein P1 (RPLP1), mouse peptidylprolyl isomerase A (PPIA), and mouse RNA ribosomal 1 (Rnr1) were chosen as the housekeeping genes (Table 1), and data were analyzed using the  $\Delta\Delta C_t$  method. The primers for hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ), and housekeeping genes used in this study were all purchased from Qiagen and are listed in Table 1. Amplification was run using a maximum of 35 cycles.

*- Protein extraction:*

The total protein content was extracted from 300 islets from each condition using M-PER Mammalian Protein Extraction Reagents and HALT™ Protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Illkirch-Graffenstaden, France). Protein concentration was determined using the Bradford micro-method (Bio-Rad, Life Science Group, Marnes-la-Coquette, France) and was expressed as mg/mL.

*- HIF-1  $\alpha$  activation (ELISA):*

HIF-1 $\alpha$  activation was quantified by TransAM™ HIF-1 (Active Motif, Belgium) following the manufacturer's instructions. 20 $\mu$ g of the total protein extraction were used to assess HIF-1 $\alpha$ . HIF dimers, which binds specifically to hypoxia response element (HRE), were immobilized in the 96-well plate. HIF dimers were detected by HIF-1 $\alpha$  antibody (dilution: 1/500) and a secondary antibody conjugated to HRP (dilution: 1/1000). The results are expressed in OD.

*- Western blotting:*

Equal protein concentrations (15 $\mu$ g) from each sample were mixed with sample buffer, loaded, separated by electrophoresis on polyacrylamide gel (Criterion™ XT 4-12%Bis Tris, Bio-Rad), and wet-transferred onto PVDF membranes. Membranes were probed with primary antibodies (4°C overnight) against cyclooxygenase-2 (COX-2) (source rabbit 1/1000e, Abcam, Paris, France), NF- $\kappa$ b p65 (source rabbit 1/500e, Cell Signaling), phospho-NF- $\kappa$ b p65 (source mouse 1/500e, Cell Signaling) and  $\beta$ -actin (source rabbit 1/2000e, Abcam, Paris, France; source mouse 1/2000e, Santa Cruz, Dallas, Texas, USA) as loading control. Secondary antibodies (1/5000e, Anti-mouse-HRP, or 1/5000e, antirabbit-HRP, Sigma) were incubated for 1 h at room temperature. Blots were developed with the Immun-Star™ WesternC™ kit (Bio-Rad) and recorded with a Chemidoc® (Bio-Rad). Densitometry analysis was performed with Image J software. Results were expressed relative to  $\beta$ -actin.

*- IL-6 and VEGF release:*

VEGF and IL-6 were quantified in islet supernatants using a rat VEGF ELISA kit (Tebu-bio, Le Perray-en-Yvelines, France) or interleukin (IL)-6 (R&D Systems®, Minneapolis, MN, USA). Results were expressed as pg/mL.

*- Statistical analysis:*

Statistical analyses were performed using Statistics software (StatSoft, Maisons-Alfort, France). Results were analyzed with one-way analysis of variance (ANOVA) followed by post-hoc Tukey honest significant difference (HSD) testing for the parametric data or

Kruskal-Wallis for the non-parametric analysis. Results are expressed as mean  $\pm$  SEM. A p value less than 0.05 was considered statistically significant.

## RESULTS

### *Islet viability*

Under hypoxic conditions for 24 hours, islet viability in a confined density 600 IEQ/cm<sup>2</sup> was improved especially in the islets bigger than 75 $\mu$ m (Figure 1A) but on the whole islet preparation the difference did not reach significance (Figure 1B) with addition of PFD (b) or ADE (d) alone, PFD+ADE (e) and HEMOXCell<sup>®</sup>+ADE (f) compared to 600 IEQ/cm<sup>2</sup> under hypoxia and confinement without molecules (a) (control condition). Nevertheless, the addition of HEMOXCell<sup>®</sup> (b) improved cell viability significantly ( $p < 0.05$ ) confirmed by a decrease of dead cells staining compared to control (a) (Figure 1A).

### *Hypoxia marker expression*

HIF-1 $\alpha$  mRNA expression (Figure 2A) and HIF-1 $\alpha$  protein translocation (Figure 2B) was significantly decreased ( $p < 0.05$ ) with all the treatments. However, the effect was emphasized with PFD+ADE and HEMOXCell<sup>®</sup> with or without adenosine for HIF-1 $\alpha$  mRNA. This observation was consistent with protein translocation. Regarding VEGF secretion in the culture medium, no difference was observed between PFD and/or ADE and the control. Only addition of HEMOXCell<sup>®</sup> with or without ADE increased significantly the secretion of VEGF (Figure 2C,  $p < 0.001$ ).

### *Inflammation*

In order to evaluate the effects of the decrease of hypoxia using oxygen carriers under confinement on the inflammatory response, we measured the relative protein expression of inflammation markers NF- $\kappa$ B and phospho-NF- $\kappa$ B, COX-2, as well as the secretion of IL-6. Despite the fact that no significant effect on NF- $\kappa$ B was observed in our conditions (Figure 3A), the trend in phosphorylation of NF- $\kappa$ B was closed to COX-2 expression. PFD alone had no impact on inflammation markers. Adenosine alone or in combination with PFD increased significantly the level of COX-2 as compared with control (Figure 3B,  $p < 0.05$ ;  $p < 0.001$ ). Independently of the presence of adenosine, HEMOXCell<sup>®</sup> significantly increased the level of COX-2 ( $p < 0.05$ ). The same pattern was obtained with IL-6; followed the secretion was increased in presence of adenosine or PFD and adenosine ( $p < 0.05$ ). IL-6 secretion was decreased by HEMOXCell<sup>®</sup> ( $p < 0.05$ ) (Figure 3C).

### *Islet functionality*

In control condition, the function was loss. Insulin secretion was not related to high glucose concentration and the stimulation index was above 1 translating the absence of insulin response. The addition of PFD, adenosine alone or mixed did not improve the response to glucose (Figure 4A). The leaking of insulin was independent of the presence of glucose ( $p < 0.05$ ;  $p < 0.001$ ), and the stimulation index below 1 (Figure 4B). The addition of HEMOXCell<sup>®</sup> in the medium, independently from the presence of adenosine, improved significantly the response to glucose stimulation by an increase in insulin release ( $p < 0.001$ ), reflected by a stimulation index between 2 and 4 ( $p < 0.05$ ). Moreover, figure 4B showed that

70% of islet preparations were functional in the presence of HEMOXCell<sup>®</sup>, 62% in presence of HEMOXCell<sup>®</sup> combined with adenosine but no functionality in the other conditions.

## DISCUSSION

In the present study, we have shown that the addition of PFD or HEMOXCell<sup>®</sup> with or without adenosine maintained islet viability by decreasing hypoxia. However, inflammation was not reduced by PFD or adenosine, which likely led to the inability to restore islet function. On the other hand, the use of HEMOXCell<sup>®</sup> decreased inflammation and restored islet function under confinement and hypoxia.

Islets are highly sensitive to hypoxia [7]. This sensitivity is emphasized in a bioartificial pancreas in which the level of O<sub>2</sub> is very low [26]. In hypoxia, because of ATP depletion, islet function is impaired and islets are exposed to inflammatory factors and reactive oxygen species (ROS) [10], which is detrimental to the function of the bioartificial pancreas. In order to address this oxygen issue, a molecular approach was developed in the present work by using PFD and HEMOXCell<sup>®</sup>.

Confined islets cultured in vitro in the presence of PFD and HEMOXCell<sup>®</sup> without adenosine exhibited a decrease in HIF-1 $\alpha$  mRNA expressions and protein levels, attesting to the positive effect of these compounds on hypoxia. The use of perfluorocarbons [8] and hemoglobin [12, 27] have been shown to be effective for islet isolation, transplantation, and encapsulation [11, 17]. The effect of the two molecules in hypoxic conditions appears to be different.

The two molecules act differently in terms of oxygen release, which can explain the differences observed on their effects on islets. The carrying capacity of PFC is directly proportional to the partial pressure of oxygen [28]. Oxygen diffuses to reach equilibrium between PFC and the ambient atmosphere. The fact that oxygen is hosted in PFC, without any molecular links, makes it a reservoir for oxygen. In our conditions of hypoxia for 24 hours, the partial pressure of oxygen was around 55% of saturation after bubbling and only 0.5% after the culture period. The release of oxygen from PFC must have been rapid since it followed a linear relation (Henry's law [29]). There is no competition between PFC and the cells since there are no links between PFC and O<sub>2</sub>.

In contrast, O<sub>2</sub> is linked to iron in the presence of hemoglobin. The link depends upon the affinity of O<sub>2</sub> for the binding site, the partial pressure of O<sub>2</sub>, and the P50 of hemoglobin. P50 of hemoglobin is the critical parameter that determines its effect in a hypoxic environment. For instance, when a hemoglobin with a P50 of 26 mmHg is placed in an environment of 7 mmHg O<sub>2</sub>, only 10% of its O<sub>2</sub> binding sites are saturated. In myoglobins with a P50 of 2 mmHg, 78% of the binding sites will be saturated with O<sub>2</sub> at the same partial pressure [12, 30]. The P50 is the indicator of O<sub>2</sub> affinity for the binding sites, with the lowest the P50 having the strongest affinity.

We chose hemoglobin with a P50 of around the partial pressure in the bioartificial pancreas in order to compensate for the hypoxic environment. HEMOXCell<sup>®</sup> has a P50 of 37 mmHg at 37°C, which means that for partial pressures of oxygen at lower than 37 mmHg, HEMOXCell<sup>®</sup> releases oxygen against the gradient. The release follows a sigmoid pattern, meaning that hemoglobin buffers the oxygen partial pressure between two limits. The release is dependent on the partial pressure gradient but within limits of the specific hemoglobin [29].

After bubbling, the PO<sub>2</sub> in the medium was 52% oxygen saturation compared to 11% saturation after 24 hours in hypoxia.

Despite this difference, both PFD and HEMOXCELL<sup>®</sup> appeared to be able to decrease hypoxia markers, even though 24 hours was the maximum limit for PFC to be effective. The advantage of HEMOXCell<sup>®</sup> over PFD could be the intrinsic superoxide dismutase (SOD) activity, which eliminates the presence of ROS and decreases inflammation [31]. This is consistent with a previous study on SOD activity in grafted hemoglobin [30]. ROS are known as major activators of inflammation and are necessary for inducing immune reactions and defenses; however, a major stressor can overwhelm the system and trigger a detrimental pathway. We have shown in previous studies that hypoxic conditions combined with confinement triggered inflammatory pathways by inducing of COX-2 mRNA expression [10].

In this study, we observed that the addition of PFD alone had no impact on inflammatory markers; however, the addition of adenosine enhanced inflammation by causing the activation of COX-2, NF- $\kappa$ B, and the subsequent IL-6 release. Surprisingly, despite the activation of COX-2 in HEMOXCell<sup>®</sup> treatment conditions, IL-6 secretion was reduced. Release of cytokines is a major issue in islet transplantation and more so in solid organ transplantation, since it can trigger graft rejection [32, 33]. The decrease in IL-6 secretion, most likely related to the SOD activity of HEMOXCell<sup>®</sup> [24, 31, 34], would be a major advantage for the tolerance of bioartificial pancreas in vivo. The same kind of discrepancy was observed regarding VEGF secretion; despite a decrease in HIF expression, VEGF was significantly increased in the presence of HEMOXCell<sup>®</sup>. Contrary to PFC, hemoglobins interact with cells and are able to induce signaling pathways. For instance, it has been shown in macrophages that hemoglobin can activate the receptor CD163, which has anti-inflammatory properties via activation of heme-oxygenase 1 [35, 36]. Thus, it is possible that the increase in VEGF or the decrease of IL-6 observed in presence of HEMOXCell<sup>®</sup> are independent from oxygenation, but are instead activated via other pathways.

In our results, the decrease in HIF-1 $\alpha$  was not correlated with VEGF release as shown by Forsythe et al. [37]. However, it is possible that the 24-hour time point used in this study was too early to see the effects of hypoxia on VEGF secretion. In contrast, the increase in VEGF release by the addition of HEMOXCell<sup>®</sup> without adenosine could be attributed to the addition of this oxygen carrier. Some studies have suggested that hemoglobin is implicated in tissue factor (TF) upregulation, resulting in upregulation of VEGF expression and secretion [38, 39], as we observed here.

In conclusion, we have shown that the supply of HEMOXCell<sup>®</sup> without adenosine in confined islets restored islet glucose-sensing only after 24 hours of incubation under hypoxic conditions. The future direction of this study will be to validate the efficiency of HEMOXCell<sup>®</sup> to preserve the viability and function of encapsulated islets in vivo or of long-term surrogate cells.

## **NOMENCLATURE**

ATP – adenosine triphosphate, Rplp1 – ribosomal protein P1, Ppia – peptidylprolyl isomerase A, Rnr1 – RNA ribosomal 1, Hif-1 $\alpha$  – hypoxia-inducible factor 1 alpha, Cox-2 – cyclooxygenase-2, IEQ – islet equivalent, PFC – perfluorocarbons, PFD – perfluorodecalin, VEGF – vascular endothelial growth factor, PO<sub>2</sub> – oxygen partial pressure

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## DUALITY OF INTEREST

The authors declare that F. Z. is the founder and hold stock in HEMARINA SA, which produces the substance being investigated. V. P. is an employee of HEMARINA SA and doesn't hold stock.

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## TABLE LEGENDS

**Table 1: qPCR primers**

## FIGURE LEGENDS

**Fig. 1.** Effect of oxygen carriers on viability of islets cultured under hypoxia and confinement. (A) Islet viability percentage in 600IEQ/cm<sup>2</sup> 24 hours in culture under hypoxic (PO<sub>2</sub> 15 mmHg) conditions in the presence of PFD (10%), Adenosine (1mM), PFD (10%) and adenosine (1mM), HEMOXCell<sup>®</sup> (50µg/mL), HEMOXCell<sup>®</sup> (50µg/mL) and Adenosine (1mM). (B) Islet viability staining. Green staining (calcein AM) indicates the cells are alive, while red staining (EthD-1) indicates that the cells are dead. Scale bars = 100 µm. \*p<0.05 vs. control.

**Fig. 2.** Effect of oxygen carriers on hypoxia expression in islets cultured under hypoxia and confinement. Levels of hypoxia-inducible factor 1 alpha (HIF-1α) mRNA expression (A) and protein expression (B) after 24 hours in culture under hypoxia (PO<sub>2</sub> 15 mmHg) in 600IEQ/cm<sup>2</sup> in the presence of PFD (10%), Adenosine (1mM), PFD (10%) and adenosine (1mM), HEMOXCell<sup>®</sup> (50µg/mL), HEMOXCell<sup>®</sup> (50µg/mL) and adenosine (1mM). (C) VEGF

secretion measured in islet medium after 24 hours in culture in the presence of PFD (10%), Adenosine (1mM), PFD (10%) and adenosine (1mM), HEMOXCell<sup>®</sup> (50µg/mL), HEMOXCell<sup>®</sup> (50µg/mL) and adenosine (1mM). Data are presented as mean ± SEM of nine independent experiments. \* p<0.05 vs. control, \*\* p<0.01 vs. control, \*\*\* p<0.001 vs. control.

**Fig. 3.** Oxygen carrier effect on inflammation of hypoxic and confined islets. Levels of phospho-NF-κB/NF-κB (A) and COX-2 (B) protein expression in 600IEQ/cm<sup>2</sup> in culture under hypoxic (PO<sub>2</sub> 15 mmHg) conditions with or without molecules. (C) IL-6 secretion measured in islet medium after 24 hours in culture under the specified conditions. Data are presented as mean ± SEM of nine independent experiments. \* p<0.05 vs. control, \*\*\* p<0.001 vs. control.

**Fig. 4.** Effect of oxygen carrier on function of islets under hypoxia and confinement. (A) Insulin response to glucose stimulation was measured after 24 hours in hypoxia in the presence of PFD (10%), Adenosine (1mM), PFD (10%)and adenosine (1mM), HEMOXCell<sup>®</sup> (50µg/mL), HEMOXCell<sup>®</sup> (50µg/mL) and adenosine (1mM).Islets were stimulated with a 22.2mM glucose KREBS solution. Data are presented as mean ± SEM of nine independent experiments. (B) Stimulation index represents the ration between insulin secreted in stimulation condition (22.2mM) and the first basal condition (4.4mM). Stimulation index of functional islet is above 1. Data are presented as dots. Each dots represent the index of stimulation of one islet preparation. \*p<0.05 vs. 22.2 mM glucose in control, \*\*\*p<0.001 vs. 22.2 mM glucose in control, \*p<0.05 vs. control.

## TABLE

**Table 1**

Primers	Reference
Rn_Rnr1_1_SG QuantiTect primer assay	QT00199374
Rn_Ppia_1_SG QuantiTect primer assay	QT00177394
Rn_Rplp1_2_SG QuantiTect primer	QT01745625
Rn_Hif1a_1_SG QuantiTect primer assay	QT00182532

**FIGURES**

**Fig 1**

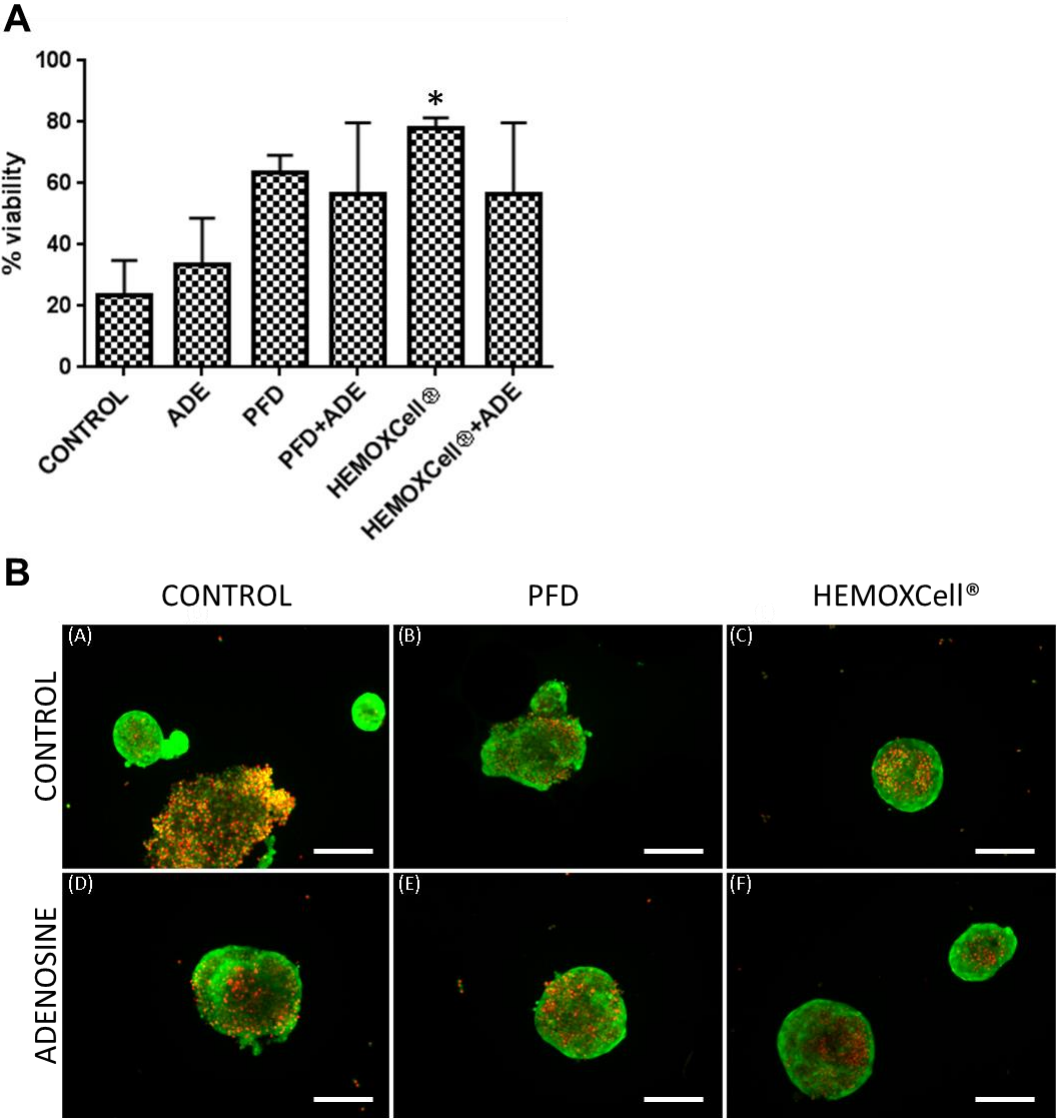


Fig 2

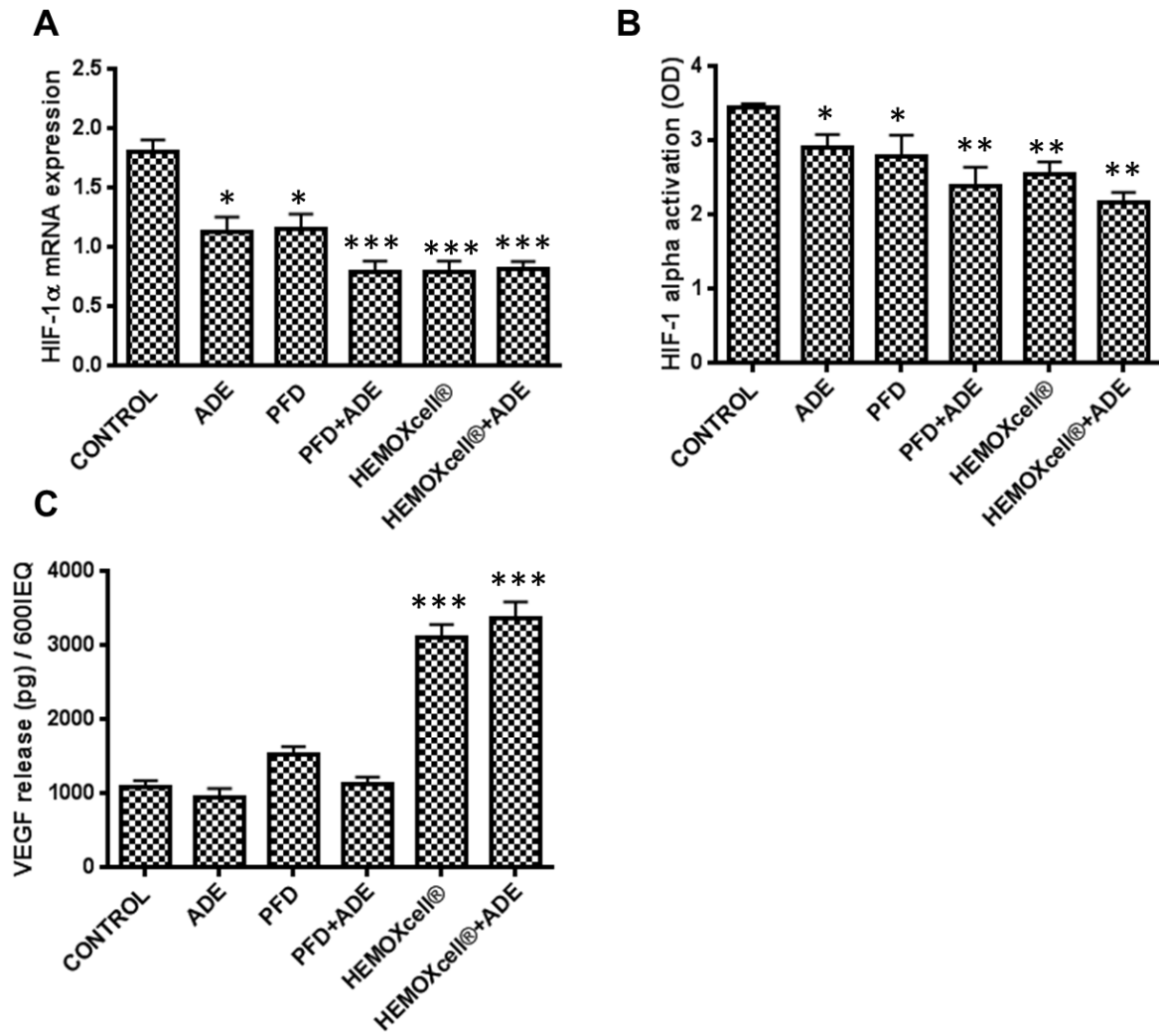


Fig 3

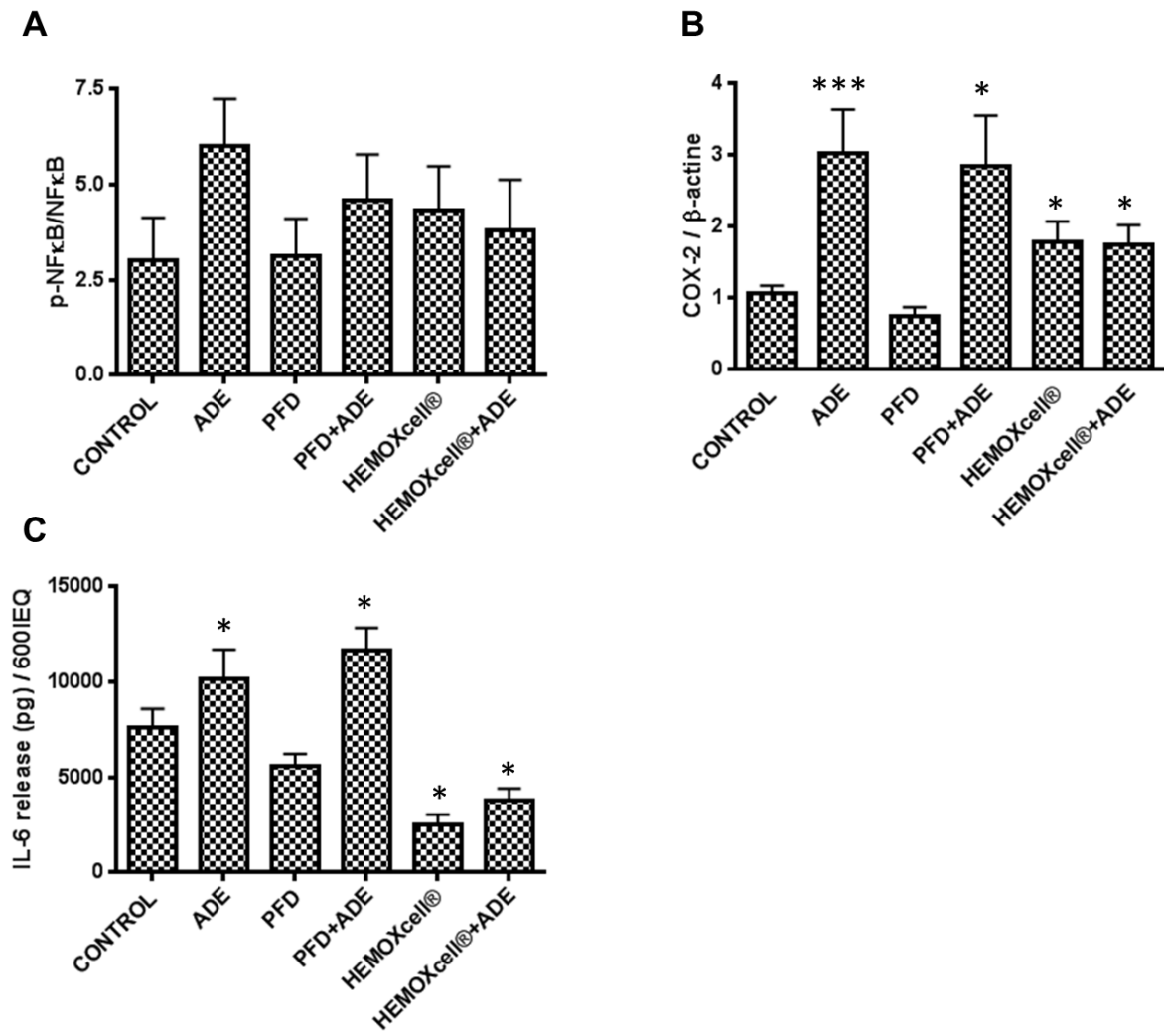
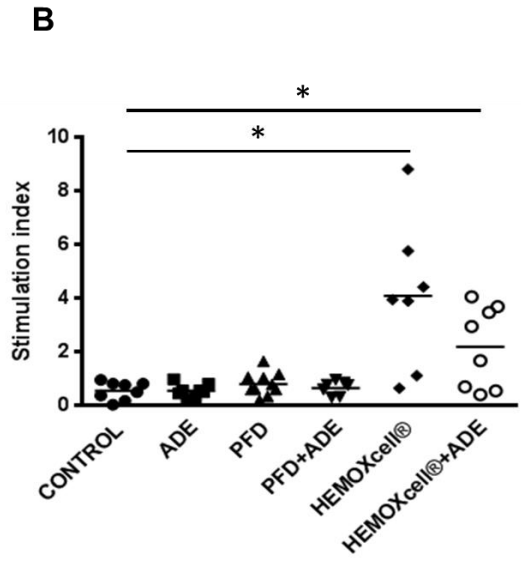
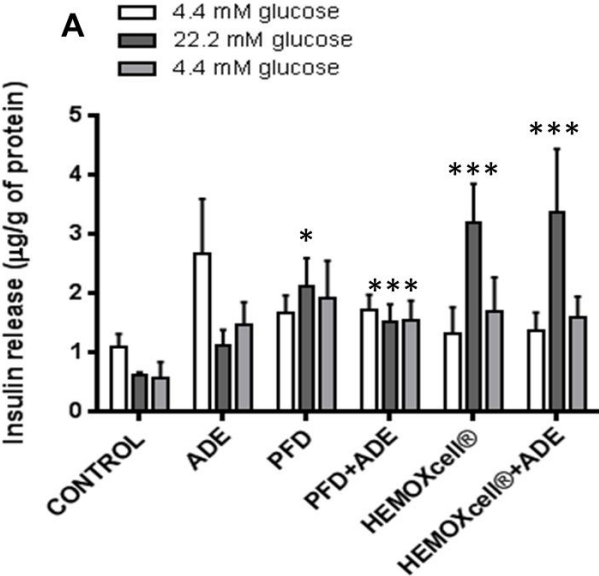


Fig 4



## 4.3 Improvement of inflammation in encapsulated islet

### 4.3.1 Introduction

In the first studies, we showed that inflammation is also one of the major problems for encapsulated islets survival. It is related to hypoxia levels and confinement. We showed an increase in COX-2 mRNA expression in islets which could enhance the increase in IL-6 synthesis and release [205]. Therefore, in this part of the work the aim was to address inflammation by challenging anti-inflammatory molecules on islet in order to improved their viability and function under confinement (600IEQ/cm<sup>2</sup>) and hypoxia (PO<sub>2</sub> = 15 mmHg).

Hypoxia leads to activation inflammation pathways driven by NF-κB , highly deleterious for islets [176]. The activation of NF-κB transcription factor induces target genes expression as MCP-1, cytokines as Interleukin-6 (IL-6) and COX-2 which is implicated in prostaglandins (PGE<sub>2</sub>) synthesis [172]. The overproduction of PGE<sub>2</sub> was showed to inhibit glucose stimulated insulin secretion [181] and thus to lead to cell dysfunction in isolated rat and human islets [188].

Different strategies have been developed over the last decades to decrease islet inflammation. For instance, blockade of the NF-κB pathway in islets prior transplantation by treatment with NF-κB inhibitors [206, 207] or antioxidants [208, 209] can improve islet graft function in experimental models. Indeed, it has been shown that NF-κB-dependent factors including nitric oxide synthase and cyclooxygenase-2 (COX-2) impact negatively in glucose stimulated insulin release and can induce islet apoptosis [175]. Schneider *et al.* reported that short-term systemic delivery of steroids and antifibrotic drugs can transiently inhibit the recruitment of inflammatory cells, and improve the protein secretion function of the encapsulated islets [210].

Targeting this signaling pathway, we could use on one hand Ibuprofen which inhibits the production of PGE<sub>2</sub> by competition with the acid arachidonic enzymatic binding site of the COX-2 [211]. On the other hand, interleukin 10 (IL-10) decreases activation of NF-κB and activate anti-inflammatory genes via the activation of IL-10 receptor and STAT protein. Moreover, IL-10 exhibited inhibitory effects on pro-inflammatory cytokines during acute inflammation [212] and a protection of pancreatic β cells [213].

Other important transcription factor implicated in inflammation could be targeting: it is CREB (cAMP response element binding protein). It regulates a wide variety of genes by binding to cyclic AMP response element (CRE) in the promoter regions of several pro-inflammatory genes such as COX-2 and IL-6. The use of tat-CREB peptide, which protects CREB form proteasome degradation, could maintain glucose homeostasis and β cell survival by preserving glucose sensing, insulin gene transcription and insulin secretion [214] in hyperglycemic environment. Protection of CREB from degradation in colorectal carcinoma (epithelial cells) exposed hypoxia using tat-CREB peptides improved cell survival [178].

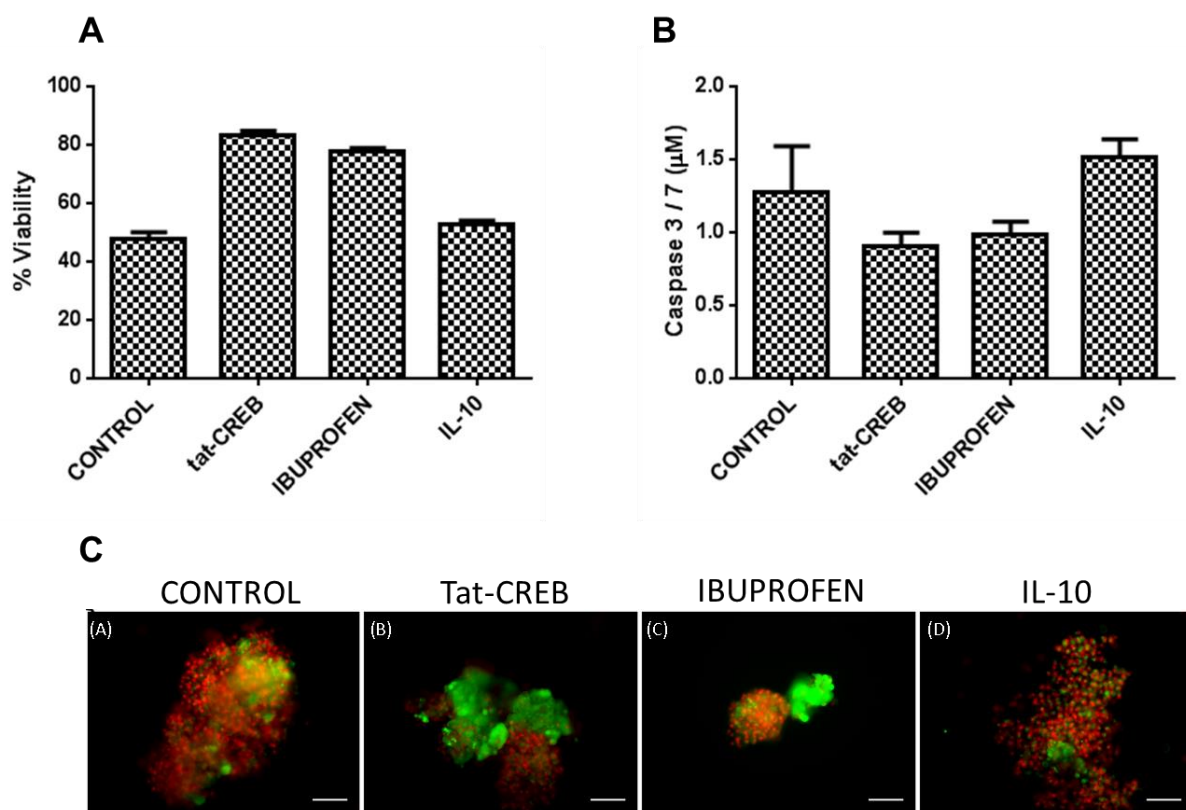
In order to decrease pro-inflammatory status of hypoxic islets, Ibuprofen, IL-10 and the novel peptide tat-CREB peptide, were tested in our conditions to decrease inflammation markers and restore islet function.



### 4.3.2 Results

- **Islet Viability**

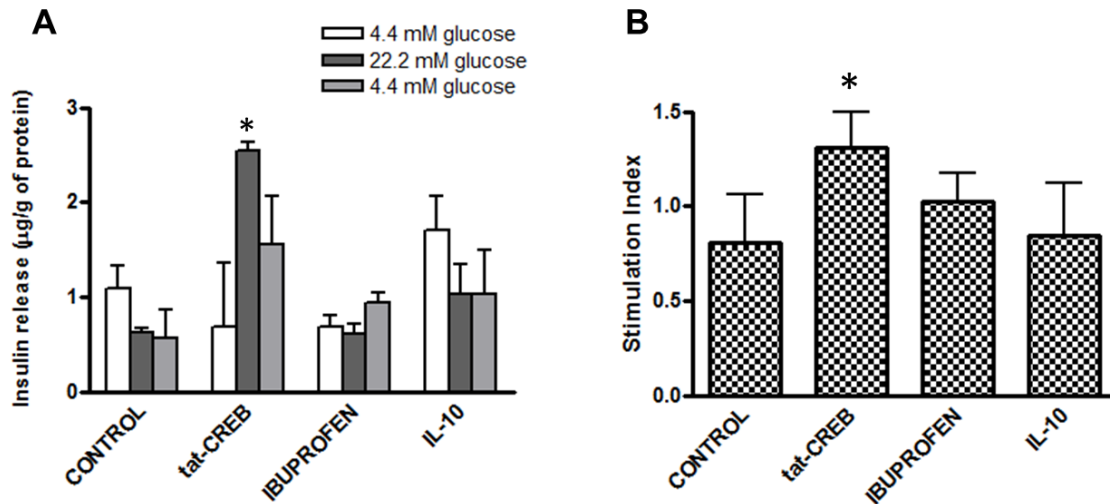
Under hypoxic conditions, the addition of tat-CREB peptide and Ibuprofen had an impact in the viability of islets cultured for 24 hours in a confined density 600 IEQ/cm<sup>2</sup> (**Fig. 4-3A**). Those results were supported by the activity levels of caspase 3/7 that seemed decrease the activation of this apoptotic marker in addition of tat-CREB peptide and Ibuprofen (**Fig. 4-3B**). Nevertheless, the addition of tat-CREB peptide (B) showed less dead cells staining than Ibuprofen (C) and IL-10 (D) compared to control condition (A), improving cell viability (**Fig. 4-3C**).



**Figure 4-3 Islet viability. (A) Islet viability percentage in 600IEQ/cm<sup>2</sup> with or without molecules after 24 hours in culture under hypoxic (PO<sub>2</sub> 15 mmHg) conditions. (B) Apoptotic marker caspase 3/7 activation levels. (C) Representative fluorescein diacetate/propidium (FDA/PI) stained images of islets. Green staining (FDA) indicates the cells are alive, while red staining (PI) indicates that the cells are dead. Scale bars = 100 μm.**

- **Islet functionality**

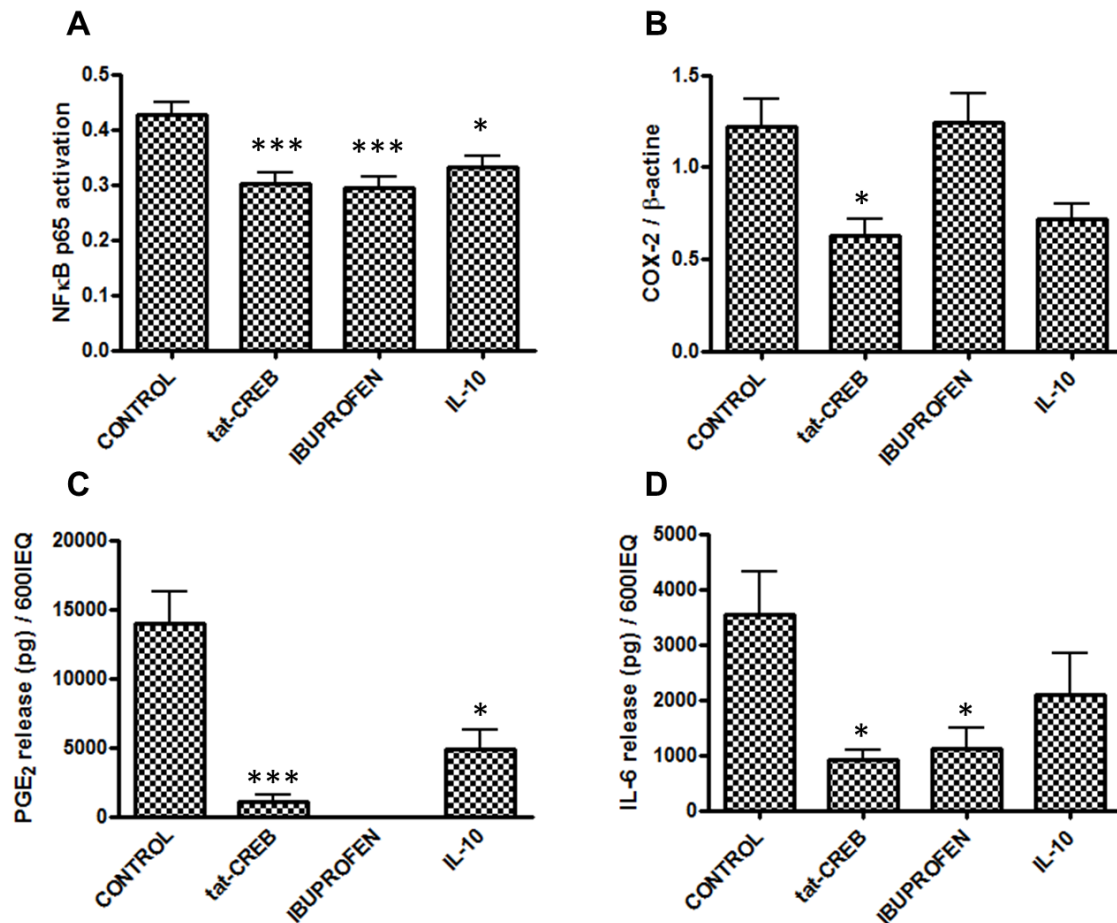
Concerning the insulin secretion under hypoxia, addition of tat-CREB peptide in 600 IEQ/cm<sup>2</sup> showed a restoration of cell function as their stimulation indices were significantly over 2 (**Fig. 4-4B**,  $p < 0.05$ ) and the insulin release in the stimulation solution was significantly increased compared to control condition (**Fig. 4-4A**,  $p < 0.05$ ). We observed an absence of function restoration with the addition of Ibuprofen and IL-10 (**Fig. 4-4A**) with the indices being reduced to less than 1 (**Fig. 4-4B**) as the control.



**Figure 4-4 Islet functionality. (A) Islets respond to increasing glucose concentrations by inducing insulin secretion. (B) Insulin secretion represented by the stimulation index. Data are presented as mean  $\pm$  SEM of nine independent experiments. \* $p < 0.05$  vs. 22.2 mM glucose in control, \* $p < 0.05$  vs. control.**

- **Pro-inflammatory status of pancreatic islets**

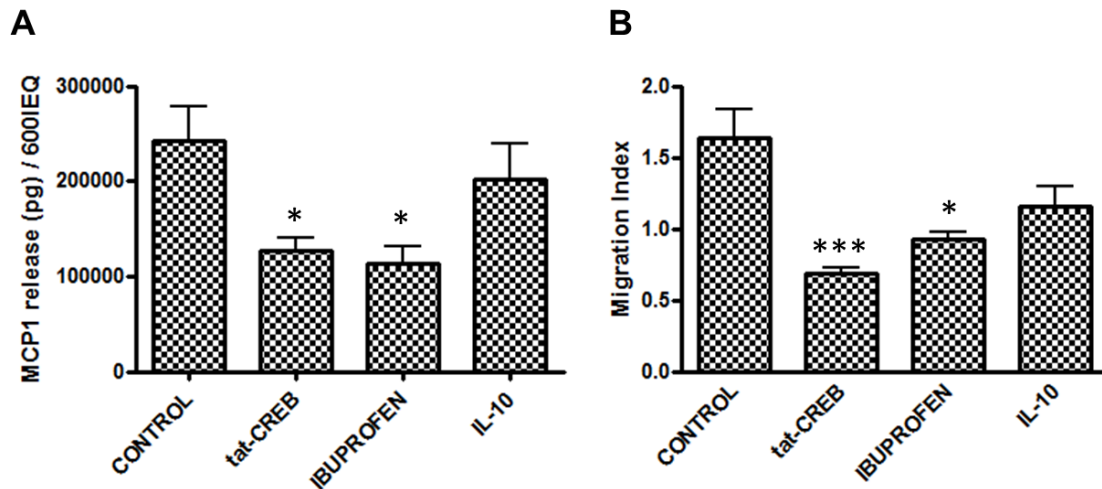
In order to evaluate the effects of the anti-inflammatory drugs on islets confined and hypoxic, we measured the NF- $\kappa$ B pathway in which expression of pro-inflammatory markers as COX-2 and PGE<sub>2</sub> as well as the secretion of IL-6 were implicated. We observed that the addition of tat-CREB peptide, Ibuprofen and IL-10 decreased significantly the activation of NF- $\kappa$ B p65 (**Fig. 4-5A**,  $p < 0.05$ ;  $p < 0.001$ ) compared to control. Although IL-10 showed a decrease in COX-2 expression ( $p < 0.05$ ), only the addition of tat-CREB peptide showed a significant downregulation of COX-2 protein expression (**Fig. 4-5B**) as compared to control. Furthermore, release of PGE<sub>2</sub> was significantly decreased by tat-CREB peptide and IL-10, with a total inhibition with Ibuprofen addition (**Fig. 4-5C**,  $p < 0.05$ ;  $p < 0.001$ ). Following the same pattern than PGE<sub>2</sub> release, IL-6 secretion appeared to be downregulated with the addition of IL-10 ( $p < 0.05$ ), with a significantly decreased by tat-CREB peptide and Ibuprofen addition (**Fig. 4-5D**,  $p < 0.05$ ) as compared to control condition.



**Figure 4-5 Pro-Inflammation.** Levels of NF $\kappa$ B p65 activation (A) and COX-2 (B) protein expression in 600IEQ/cm<sup>2</sup> in culture under hypoxic (PO<sub>2</sub> 15 mmHg) conditions with or without molecules. PGE<sub>2</sub> (C) and IL-6 (D) secretion measured in islet medium after 24 hours in culture under the specified conditions. Data are presented as mean  $\pm$  SEM of nine independent experiments. \*  $p < 0.05$  vs. control, \*\*\*  $p < 0.001$  vs. control.

- **Chemokines activation and macrophages recruitment**

Levels of the inflammatory chemokine MCP-1 were also evaluated. We observed a significant decrease in MCP-1 release with the addition of tat-CREB peptide and Ibuprofen as compared to control (**Fig. 4-6A**,  $p < 0.05$ ). Those results were supported by the chemotaxis assay represented by the macrophages migration index, which showed a decrease in the migration index with the addition of tat-CREB peptide and Ibuprofen (**Fig. 4-6B**,  $p < 0.05$ ,  $p < 0.001$ ) as compared to control.



**Figure 4-6 Chemokines activation and macrophages recruitment. (A) MCP-1 secretion measured in islet medium after 24 hours in culture in 600IEQ/cm<sup>2</sup> in culture under hypoxic (PO<sub>2</sub> 15 mmHg) conditions with or without molecules. (B) Chemotaxis assay performed in islets medium under the specified conditions represented by the migration index. Data are presented as mean ± SEM of nine independent experiments. \*  $p < 0.05$  vs. control, \*\*\*  $p < 0.001$  vs. control.**

### 4.3.3 Discussion

Inflammation is one of the main causes of  $\beta$  cell loss in the bioartificial pancreas due to lack of nutrients and oxygen. In the present study, we have shown that the addition of tat-CREB peptide decreased inflammation with restoration of islet function under confinement and hypoxia (15 mmHg), whereas well described anti-inflammatory compound such as Ibuprofen or IL-10 have an impact on inflammation but failed for the restoration of function.

Target of IL-10 and Ibuprofen are different with an advantage in inhibition of inflammation pathway for Ibuprofen over IL-10. Ibuprofen prevents the production of PEG2 and NF- $\kappa$ B DNA binding [211, 215]. IL-10 inhibits NF- $\kappa$ B in two different ways: by inhibiting activation of I $\kappa$ B Kinase and by inhibiting NF- $\kappa$ B DNA binding activity [216, 217]. Addition of Ibuprofen does not affect COX-2 protein levels but protein activity revealed by PEG2 production. However, studies have reported that COX-2 is downregulated at transcriptional and post-transcriptional levels in presence of IL-10 as we have seen in our results [191]. Chemotaxis was impacted differently by the three molecules. Ibuprofen as tat-CREB peptide decreased MCP-1 secretion and the subsequent macrophage migration. At the opposite, IL-10 did not have any effect.

Tat-CREB peptide has never been tested as anti-inflammatory molecule. As it protects CREB from degradation and because CREB is involved in numerous survival pathways it seemed interesting to study effect of such peptide on inflammation generated by hypoxia. Ollivier *et al.* have demonstrated that elevated intracellular cAMP, in response to inflammation [218], and activation of the protein kinase A (PKA) inhibits NF- $\kappa$ B-mediated transcription [219]. PKA signaling pathway is responsible of CREB phosphorylation and the subsequent recruitment of the transcriptional coactivator CREB-binding protein (CBP) [220]. CBP acts as an integrator of multiple signal transduction pathways and that competition for limiting amounts of CBP can result in the inhibition of transcription factor activity [221]. In our study, protection of CREB from degradation triggers a competition between CREB and NF- $\kappa$ B for limiting amounts of CBP resulting in a NF- $\kappa$ B decrease corresponding with those findings by Parry *et al.* The downregulation of COX-2 protein levels in presence of tat-CREB peptide is a consequence of the reduction in NF- $\kappa$ B -mediated transcription [181].

Reduction in NF- $\kappa$ B translocation has been correlated in the literature with a decrease in IL-6 and MCP-1 in islets culture medium [193]. This is also in line with our results that showed a decrease in MCP-1 release, directly correlated with a decrease in macrophages migration due to the capacity of MCP-1 to induce chemotaxis [199]. However, inhibition of chemokine production including MCP-1 by IL-10 [222], is not supported by our results that showed no changes in MCP-1 and macrophages stimulation index. This apparent contradiction can be readily explained, since macrophages and endothelial cells present in the islet preparations could be the cause of the MCP-1 release. If so, the MCP-1 expression must be balanced with the IL-10 inhibition [223]. Addition of tat-CREB peptide was also implicated in a decrease in MCP-1 release and macrophages migration as a consequence of NF- $\kappa$ B translocation reduction [193]. In the case of the application of such treatment for encapsulated islets, it would highly be beneficial for islets to decrease to chemokines and cytokines secretions.

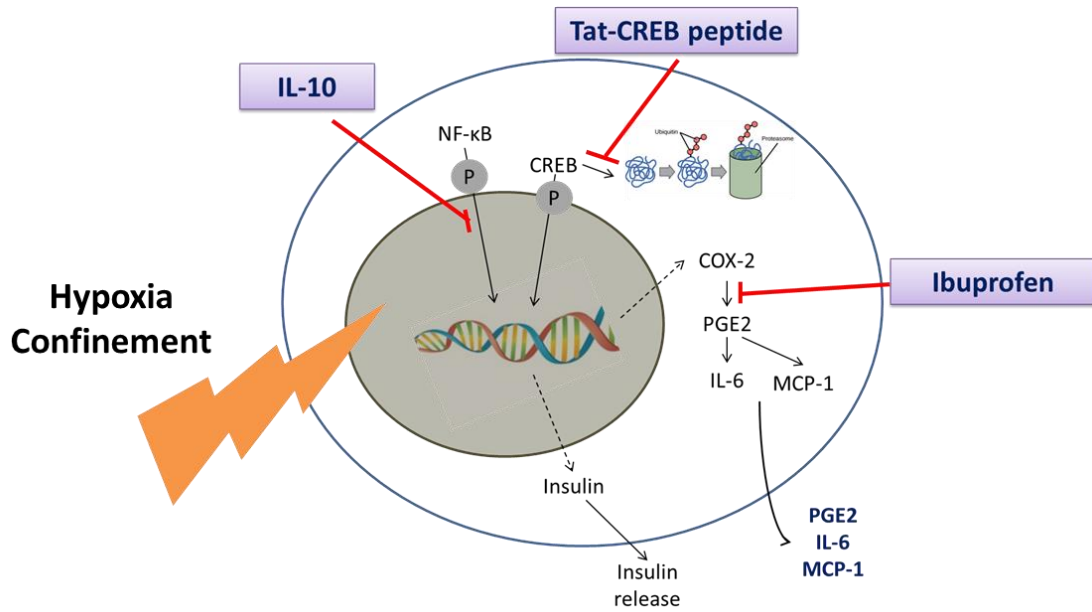
Moreover, in terms of islet function and viability the only anti-inflammatory drug has protected islet function and improved islet viability was the addition of tat-CREB peptide. In freshly isolated human islets, expression of cytokines as IL-6 and MCP-1 were markedly elevated together with impaired insulin secretion function before transplantation [193]. Recently, it has been shown that diabetic patients transplanted with islets that produce high

levels of MCP-1 were not able to induce long term insulin independence in contrast of those who receive islets that produces low levels of MCP-1 [198]. Studies with IL-10 in islets transplantation showed a protection of pancreatic  $\beta$  cells and glucose stimulation insulin secretion [212] and studies with Ibuprofen in different type of cells showed a decrease in pro-inflammatory markers as we have seen in our study [211, 215, 224]. However, those experiments were not performed under hypoxic condition which is the principal cause of loss of islets functionality and reduction in islet viability [205] correlated with previous results.

The present work exhibited that the decrease of inflammation was important to preserve islet viability but not the function. Cytokine and chemokine secretion were not responsible for the loss of function and the major issue is in fact hypoxia.

To confirm the role of tat-CREB as a novel anti-inflammatory drug, further studies need to be done. Future experiments as levels of CREB, pCREB, GLUT-2 (glucose transporter in  $\beta$  cells) and glucose kinase will be performed in islets under hypoxia and confinement. The study of the degradation pathway of glucose will be interesting to understand how tat-CREB permits the preservation of islet function. As activation of CREB resulted in an induction of bcl-2 gene expression involved in survival pathway [177], levels of bcl-2 and caspase-3 will be also assessed to explain islet survival.

The advantage of tat-CREB is the maintenance of function while inflammation is decreased. The impact of tat-CREB could be emphasizing by other molecules inhibiting inflammation further. For instance, TPL-2 (Tumor Progression Locus 2), or Cot for Cancer Osaka Thyroïde or MAP3 kinase 8 (MAP3K8), is a serine-threonine kinase with an important physiological role in inflammation signaling. TPL-2 is released from the inactive complex by IKK, therefore it is closely link to NF- $\kappa$ B activation. TPL-2 is involved in activation MAPK, JUNK, and ERK1/2. Using a specific inhibitor of TPL-2 in combination with tat-CREB, the impact on cell survival could be very important, as shown by Varin *et al.* The impact of TPL-2 inhibitor and exenatide, prevented of cytokine-induced death and dysfunction in human islets [225] (**Fig. 4-7**).



**Figure 4-7 Impact anti-inflammatory molecules tested in islets**

In conclusion, we have shown that supply of tat-CREB peptide in confined islets restored islets function and decrease pro-inflammatory markers after 24 hours of incubation under hypoxic conditions.

## 5 DISCUSSION, CONCLUSION AND PERSPECTIVES

L'une des options disponibles pour un nombre limité de diabétiques de type 1 est une greffe de pancréas entier, ce qui est réalisé depuis 1966. Depuis le début des enregistrements, plus de 40.000 greffes de pancréas ont été enregistrées dans le registre de transplantation du pancréas international (IPTR), la majorité aux Etats-Unis. On estime que plus de 7.000 transplantations pancréatiques ont été réalisées en Europe à ce jour. Le nombre de patients inscrits sur la liste de transplantation actif au Royaume-Uni pour un PTA (transplantation de pancréas seul), SPK (pancréas et rein simultané) et IT (îlots seuls) a considérablement augmenté au cours des dix dernières années, passant de 132 patients en 2005 à 270 patients en 2014. Le nombre de donneurs de pancréas et de transplantation a également augmenté régulièrement, passant de 118 donneurs résultant à 86 transplantations en 2004-2005, à 456 donneurs et 246 greffes en 2013-2014 [226].

Une amélioration de la survie des patients et des greffons ont été observés s'accompagnant d'une diminution des complications microvasculaires spécifiques des diabétiques. Pour la transplantation simultanée rein/pancréas, la survie des patients a maintenant atteint plus de 96% à 1 an après la transplantation, et plus de 83% à 5 ans après la transplantation. La meilleure survie du greffon est vue dans les cas de transplantation rein/pancréas avec 86% et 93% de fonction du greffon rénal à 1 an. Avec la transplantation pancréas après le rein, la fonction du greffon pancréatique atteint 80%, et avec la transplantation de pancréas seul, la fonction de la greffe de pancréas est de 78% à 1 an [227]. La fonction de la greffe est déterminée comme étant le critère de survie du greffon par le IPTR. La demi-vie de greffons (50% fonction) est également améliorée, avec la transplantation simultanée pancréas/rein elle est 14 ans, 7 ans pour le pancréas après le rein et pour le pancréas seul elle de 7 ans. La longévité du greffon la plus importante a été observée pour la greffe simultanée pancréas/rein et pancréas seul, avec 26 ans et 24 ans respectivement.

La recherche en thérapie cellulaire offre une alternative pour le traitement des diabétiques de type 1 et comme elle est moins invasive, elle reste très attrayante, en particulier pour les patients qui sont considérés comme présentant des risques liés à l'acte chirurgical.

La transplantation d'îlots humains pour restaurer une régulation physiologique de la glycémie chez des diabétiques insulino-dépendant a commencé dans les années 1970 [228], mais ce ne fut qu'en 1989 que le premier receveur a été en mesure d'arrêter l'insuline exogène [229]. En 2000, l'équipe d'Edmonton a établi un protocole clinique permettant le restaurer l'insulino-indépendance pendant 1 an chez 7 patients. Depuis, 750 patients diabétiques de type 1 ont bénéficié de la greffe d'îlots dans un des quelques 30 centres mondiaux d'isolement d'îlots actifs au cours de la dernière décennie. En comparaison, environ 30.000 greffes de pancréas ont été menées au cours des trois dernières décennies. Malgré le nombre relativement faible des bénéficiaires des îlots, des résultats encourageants avec la transplantation d'îlots ont récemment été observés. Par exemple, le taux de 50% de patients toujours insulino-indépendant à 5 ans a été atteint ce est équivalent à la transplantation de pancréas entier dans au moins quatre centres indépendants, à savoir Edmonton, Minneapolis, Genève et Lille. Il est évident que des progrès significatifs récents dans la préparation d'îlots et de la thérapie immunosuppressive ont permis d'améliorer l'efficacité et la sécurité de la transplantation d'îlots au point qu'elle peut concurrencer maintenant la greffe de pancréas entier.

Aujourd'hui, le programme de transplantation d'îlots clinique à Edmonton est reconnu comme l'un des centres de transplantation le plus important et le plus actif dans le monde.



Après être devenu un leader avec l'une des approches les plus intégrées et réussies pour la transplantation d'îlots présentant des résultats durables et reproductibles à long terme. Une étude récente a publié une analyse transversale des résultats actuels Edmonton. Elle a montré que pour 79% des cas (sur plus de 300 greffes réalisées), la fonction du greffon était complète ou partielle. La durée médiane de l'indépendance à l'insuline était de 34,6 et 11,0 mois pour les patients ayant une fonction complète ou partielle du greffon, alors que la durée de C-peptide était de 53,3 et 70,4 mois pour ces mêmes patients [230]. Des progrès importants ont eu lieu au cours de ces dernières années grâce à la mise en œuvre de nombreuses d'études précliniques et cliniques pour tester différents agents pour permettre une meilleure immunotolérance avec des complications moindres, de nouveaux dispositifs pour fournir des îlots avec un environnement plus sûr, ainsi que de nouveaux sites de transplantation pour surmonter les limitations inhérente à l'accès intraportal courant [231].

En revanche, la perte progressive de la fonction des îlots au fil du temps en raison des processus chroniques hypoxie et inflammatoires au site de transplantation intraportal demeurent des défis non résolus dans la transplantation d'îlots. Un procédé prometteur pour éviter la nécessité d'une immunosuppression à long terme est l'encapsulation des îlots à l'intérieur d'une membrane immunoprotectrice [146].

Le développement d'un pancréas bioartificiel a impliqué la microencapsulation ou la macroencapsulation des îlots de Langerhans. Dans l'ensemble, les stratégies de microencapsulation n'ont pas permis de reverser systématiquement le diabète autre que dans les modèles de rongeurs. Un revêtement conformationnel d'îlots est étudié comme un moyen de contourner les problèmes de l'épaisseur de la capsule (générant de l'hypoxie) et de la diffusion provoquée par l'épaisseur de la capsule dans un modèle de microencapsulation traditionnelle. Des résultats plus probants, cependant, ont été obtenus dans les grands mammifères avec macroencapsulation, sous la forme de sachets, de feuillets, et de dispositifs plus ou moins sophistiqués. De plus, les dispositifs de macroencapsulation peuvent être chargés avec différentes sources de cellules illimitées capables de sécréter de l'insuline d'une manière régulée, comme des îlots xénogéniques souches ou tissus provenant d'une cellule, ce qui pourrait résoudre le problème de la pénurie d'organes et d'offrir un remède à tous les individus diagnostiqués avec diabète de type 1 [232]. Les îlots de porcs néonataux encapsulés dans le dispositif TheraCyte<sup>®</sup> restaient viables jusqu'à 8 semaines après la xénotransplantation en dans un modèle de singes nondiabetic [233]. De plus, la macroencapsulation d'îlots de porc dans un modèle en feuillet transplanté en sous-cutané a montré son efficacité pour le contrôle du diabète jusqu'à 1 an dans 2 primates diabétiques [234].

L'implantation du dispositif Bêta-Air<sup>®</sup> (n = 1) a montré que les îlots humains survivent et sont capables de conserver leur réactivité au glucose 10 mois après la transplantation chez patient diabétique. Cependant, en raison de la masse minimale des îlots transplantés (2,100IEQ / Kg), seuls des résultats modérés sur l'amélioration des conditions glycémiques ont été montrés [145]. D'autres stratégies possibles sont en développement et elles passent par un système artificiel complètement autonome. Il consiste en une combinaison d'une pompe à insuline portable et implantable, d'un systèmes de surveillance continue du glucose (CGMS) et d'algorithmes de contrôle en boucle fermée. Aujourd'hui disponible, le système de pancréas artificiel présente quelques limites principalement liées aux retards dans la détection du glucose et l'absorption d'insuline due au site sous-cutané. Un autre problème est la courte durée de vie des capteurs de glucose. Par conséquent les développements actuels se portent sur l'amélioration des performances du pancréas artificiel afin sur le développement d'un dispositif artificiel implantable dans le péritoine (site qui permet une meilleure biodisponibilité de l'insuline), sur de nouvelles technologies de détection du glucose, sur une amélioration de la

fiabilité, sur l'allongement de la durée de vie du matériel et sur de nouveaux algorithmes de contrôle qui reflèteraient en temps réel le taux de glucose sanguin. Les travaux récents ont permis le développement et la miniaturisation d'outils pour le remplissage artificiel des pompes, l'utilisation de matériaux fonctionnalisés, etc. ce qui devrait permettre le développement d'un pancréas artificiel totalement implantable miniaturisé. Un travail considérable est encore nécessaire pour parvenir à des solutions fiables et sûres [235].

L'objectif du développement du pancréas bioartificiel est la restauration physiologique de la sécrétion d'insuline sans traitement immunosuppresseur, ce qui permettra au jeune patient diabétique de type 1 traité de limiter les risques de complications à long terme.

L'isolement immunologique permet l'abrogation du traitement et en même temps, comme la protection fonctionne dans les deux sens, l'hôte est protégé de la greffe. Dès lors, l'utilisation de cellules souches devient possible, ce qui résoudrait les problèmes de pénuries d'organes.

Defymed a développé ces dernières années un prototype de pancréas bioartificiel conçu pour l'homme, le MAILPAN<sup>®</sup> (MACroencapsulation of PANcreatic Islets). Afin d'optimiser la survie et le fonctionnement des cellules dans le MAILPAN<sup>®</sup>, le projet nommé BIOSID fut monté (BIOartificial Secreting Insulin Device). Ce projet a été financé par la Communauté Européenne FP7, qui a validé la convention de subvention référencé: HEALTH-F2-2012-30574. BIOSID a tenté de résoudre les problèmes liés à l'encapsulation, tels que l'hypoxie et l'inflammation et diminuer le risque de rejet, par l'étude de biocompatibilité systématique du MAILPAN<sup>®</sup>.

Pour ce projet, des experts en transplantation d'îlots, en cellules souches et en encapsulation ont été regroupés pour tester les îlots, ainsi que différentes sources de cellules sécrétrices d'insuline dans la cadre du MAILPAN<sup>®</sup>.

Le prototype a été testé sur des modèles précliniques, comme les rongeurs ou les primates, avec pour objectif de développer l'un des premiers dispositifs de macro encapsulation approprié pour être implanté sur un patient diabétique de type 1.

Les différentes étapes de ce projet ont été:

- De comprendre les besoins des îlots dans le dispositif MAILPAN<sup>®</sup> pour leur survie et le maintien de leur fonctionnalité
- Développer des conditions optimales pour augmenter la survie et la fonction des îlots / des pseudoîlots de cellules Endo $\beta$ -H1 (lignée de cellule beta humaine) dans le dispositif MAILPAN<sup>®</sup>
- Valider le pancréas bioartificiel chez les primates non-humains
- Valider le pancréas bioartificiel chez les humains : phase I/II études cliniques

Dans le cadre de ce projet, le rôle du CeeD a été de travailler sur la compréhension des conditions internes du dispositif, le comportement des cellules dans de telles conditions et finalement comment améliorer le taux de survie.

En nous fondant sur les données de la littérature, nous avons déterminé le nombre des îlots nécessaires pour reverser le diabète. Dans le domaine d'encapsulation d'îlots libre, le chiffre communément accepté est de 10,000 IEQ/kg. Dans le domaine de l'encapsulation d'îlots les données sont plus éparses, la fourchette des îlots requis débute à 15,000 IEQ/kg de receveur [236] à 130,000 IEQ/cm<sup>2</sup> [237, 238]. Chez notre modèle de rat, nous avons choisi entre 10,000 et 15,000 IEQ/kg pour un homme, ce qui donne entre 5,000 et 10,000 IEQ par dispositif ou 300IEQ/cm<sup>2</sup> et 600IEQ/cm<sup>2</sup> pour un rat.

Nous avons tout d'abord testé la fonctionnalité du dispositif avec 300IEQ/cm<sup>2</sup> (5,000IEQ qui est équivalent aux exigences de la greffe d'îlots libres) *in vitro* avant toute expérience *in vivo*. Pour effectuer ce test de stimulation au glucose, les îlots ont été placés dans le dispositif en présence d'une solution à faible concentration en glucose (4.4 mmol/L glucose ce qui est lié à un niveau basal de glucose non-stimulant). Le dispositif fut ensuite incubé pendant plusieurs heures dans un Becher contenant 100mL de solution avec une concentration élevée de glucose (22.6 mmol/L glucose, concentration considérée comme stimulant la sécrétion d'insuline). Des échantillons ont été prélevés chaque heure pendant une durée de 6 heures. Le MAILPAN<sup>®</sup> a été alors transféré dans une solution à faible concentration de glucose pour plusieurs heures (en renouvelant la solution après chaque prélèvement pour pouvoir détecter la diminution). La sécrétion d'insuline a été détectée en dehors du MAILPAN<sup>®</sup> dès la première heure de stimulation avec un maximum de concentration à 2 heures. La baisse d'insuline libérée a été relevée après la deuxième heure d'incubation dans la solution à faible concentration en glucose. Avec cette expérience simple, nous avons confirmé que:

- Le glucose pouvait pénétrer le dispositif,
- Les îlots dans le dispositif pouvaient être stimulés par ce glucose et pouvaient sécréter de l'insuline
- L'insuline est capable de traverser la membrane
- Et finalement le glucose peut également sortir, ce qui peut permettre d'arrêter la stimulation des îlots.

Tout le concept de pancréas bio (îlots) artificiel (MAILPAN<sup>®</sup>) a été validé *in vitro*.

Toutefois, la survie des îlots *in vitro* dans le dispositif est limitée. Après 24 heures *in vitro* dans le MAILPAN<sup>®</sup>, très peu d'îlots ont été retrouvés, la plupart d'entre eux ayant probablement été détruits dans de telles conditions, ce qui a rendu l'analyse difficile. Dans ces conditions statiques, le dispositif ne peut pas fonctionner de façon optimale, car la diffusion au travers des membranes est limitée *in vitro*. Pour cette raison, nous avons besoin d'un modèle approprié de milieu de culture, afin de simplifier le travail et obtenir des données plus solides.

Par conséquent, nous avons besoin de connaître le niveau d'oxygène contenu dans les dispositifs implantés *in vivo*.

Ainsi, les dispositifs MAILPAN<sup>®</sup> équipés d'un spot à oxygène ont été implantés vides pancréatiques dans des rats Lewis pour des mesures d'oxygène *in vivo*. Après un mois d'implantation, les niveaux d'oxygène relevés sont autour de 15 mmHg. Toutefois, les précisions de cette méthode avaient besoin d'être améliorées. Donc, une méthode non-invasive pour les mesures d'oxygène est en développement chez DEFYMED afin d'obtenir des données plus précises. La méthode non invasive est importante pour permettre de suivre les fluctuations du PO<sub>2</sub> sur le même animal pendant l'implantation du dispositif sans cellules, une fois que les îlots ont été infusés le même jour (pour voir les variations journalières liées à l'activité des îlots). De plus la sonde qui sera utilisée est plus sensible aux niveaux bas de PO<sub>2</sub> que le spot.

Entre temps, basé sur les données *in vivo*, nous avons mis en place notre modèle de culture afin d'étudier les effets de confinement et d'hypoxie sur les îlots : 600IEQ/cm<sup>2</sup> sous 15 mmHg pendant 24 heures.

L'équilibre entre les besoins élevés d'insuline pour les receveurs (ie. un nombre élevé d'îlots) et la surface nécessaire par îlot pour une oxygénation appropriée (confinement dans un endroit restreint) [239, 240] est difficile à atteindre [241]. Nous avons clairement montré que la combinaison entre confinement et PO<sub>2</sub> bas étaient néfastes pour la survie des îlots car il y a activation de l'inflammation, de l'hypoxie et de l'apoptose. En améliorant l'apport en oxygène avec HEMOXCell<sup>®</sup> nous avons montré que le maintien de la fonction des îlots était possible, mais n'était pas lié à la diminution de l'inflammation. En effet, la diminution de l'inflammation en utilisant de l'Ibuprofène ou de l'IL-10 n'était pas suffisante pour rétablir les fonctions dans nos conditions. De plus, nous avons mis en évidence le rôle central de CREB dans le maintien des fonctions, ceci corrobore des études précédentes sur les îlots dans un contexte d'hyperglycémie [202] ou sous hypoxie [178].

D'après cette étude, plusieurs questions se posent:

- Quel est l'effet réel de l'activité intrinsèque SOD de HEMOXCell<sup>®</sup> et CREB sur le maintien des fonctions des îlots dans de telles conditions?
- Quelle est l'efficacité de HEMOXCell<sup>®</sup> à long terme? Combien de temps la molécule est-elle stable in vivo? Est-il possible de recharger HEMOXCell<sup>®</sup> avec un PO<sub>2</sub> de 15mmHg bas, et si c'est possible, y-a-t-il un risque de créer une hypoxie locale si l'oxygène a plus d'affinité pour HEMOXCell<sup>®</sup> que les îlots?
- Dans ce dispositif, sera-t-il nécessaire de remplacer HEMOXCell<sup>®</sup> sur une base régulière, afin de prolonger la survie des cellules? Sera-t-il possible de fixer HEMOXCell<sup>®</sup> sur la surface intérieure du dispositif pour une libération progressive ou une action? Ou sera-t-il possible d'utiliser une autre stratégie pour recharger HEMOXCell<sup>®</sup>?
- Une fois la viabilité et la fonction résolues à court terme, quelle sera la nouvelle stratégie afin d'améliorer la survie des îlots à long terme? Les îlots sont-ils le meilleur outil pour supprimer le diabète dans le MAILPAN<sup>®</sup>?

Des études sur des îlots sous hypoxie et confinés pendant 1 à 4 jours ont permis une réflexion à l'égard de ces questions et des futures travaux.

Pour comprendre l'importance du CREB dans de telles conditions, nous avons commencé par investiguer un peu plus loin sur d'autres cibles possibles. Nous avons tout d'abord étudié les taux de HIF-1 $\alpha$  mRNA. Cela semble positif dans notre cas, car les îlots ont maintenus leur fonction et leur hypoxie. Toutefois le lien entre CREB et HIF reste encore à identifier. De par les publications nous savons que l'hypoxie diminue CREB dans les cellules épithéliales par l'activation de sa dégradation par le protéasome et en même temps l'hypoxie active l'expression HIF [178]. Tous deux sont des facteurs de transcription liant la protéine liante complexe CREB (CBP/P300) pour initier la transcription [242]. Une hypothèse serait qu'il y ait une compétition entre CREB et HIF pour lier avec le CBP/P300. La stabilisation du CREB préviendrait HIF pour obtenir le transcriptome, et comme HIF est impliqué dans sa propre transcription ; il y aurait une baisse du messenger de HIF. Dans notre cas, la baisse du messenger de HIF était en corrélation avec la maintenance de la fonction, ce qui voudrait dire que l'expression et l'activation de HIF est délétère pour les cellules. Une autre divergence ressort de nos expériences préalables. Habituellement, les taux élevés d'ATP sont en corrélation avec une bonne viabilité et fonction de la cellule. Cependant dans nos conditions, les îlots sont viables et en fonction en présence de tat-CREB mais ils présentent des niveaux bas d'ATP. Une étude intéressante mentionnait que le glucose stimule la libération d'ATP des cellules MIN6 de manière dose-dépendante [243]. De plus, il a été démontré que la

libération d'ATP a d'importants effets autocrine et paracrine promouvant la survie des différents types de cellules [244-247]. Par conséquent ces niveaux bas d'ATP pourraient être liés au combat de la cellule pour la survie. En activant et en inhibant CREB et les voies de signalisation induites par HIF-1 $\alpha$ , l'impact que ces voies peuvent avoir l'une sur l'autre pourra être déterminé. Parallèlement, l'étude de l'ATP libéré, contenu, sa production et sa dégradation sera réalisée afin de clarifier ces observations.

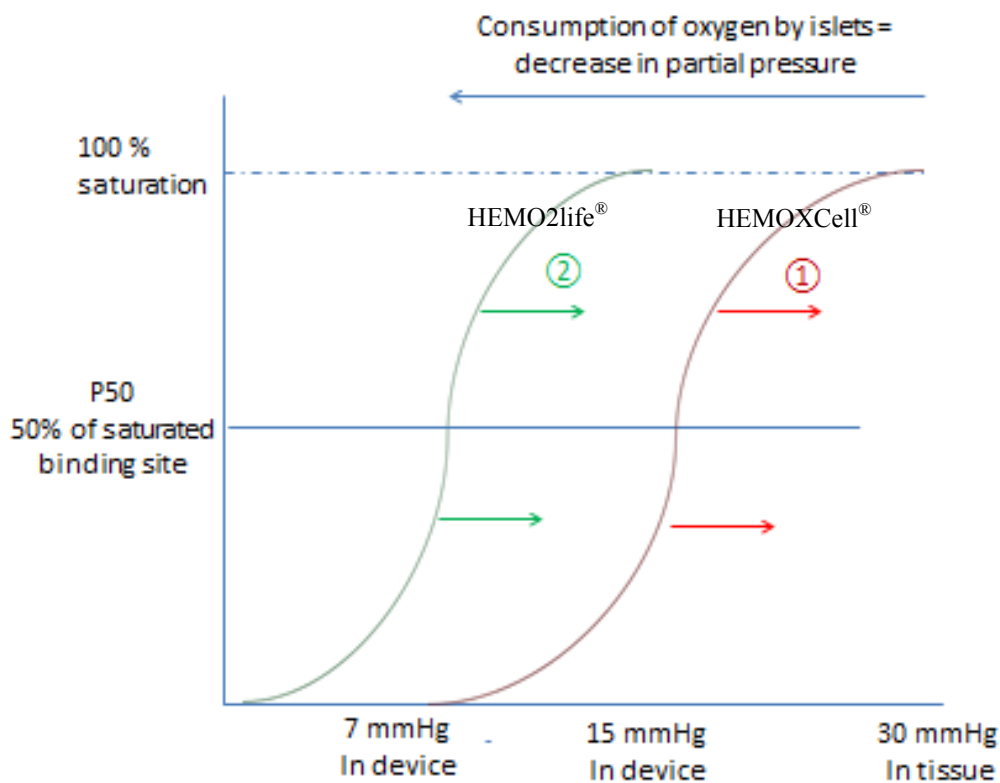
Le rôle de l'activité intrinsèque SOD de HEMOXCell<sup>®</sup> doit être déterminé. Il a été décrit que l'hémoglobine possède une activité d'oxydoréduction générant des espèces réactives de l'oxygène, tels que les anions superoxyde, hautement toxique. Comme l'hémoglobine est naturellement contenue dans les cellules qui possèdent un système antioxydant impliquant SOD et la catalase, l'utilisation d'hémoglobine sans modification comme transporteur d'oxygène serait toxique pour les cellules. Par conséquent l'association artificielle d'hémoglobine intracellulaire avec différentes enzymes antioxydantes a été développée par plusieurs équipes [248-250]. L'utilisation d'une telle hémoglobine a été testée afin de diminuer les lésions d'ischémie-reperfusion et son activité antioxydante couplée avec la diffusion d'oxygène a permis de protéger les organes des dommages. L'atout majeur d'une hémoglobine extracellulaire, tel que HEMOXCell<sup>®</sup>, c'est l'absence de modifications artificielles pour introduire une l'activité antioxydante, qui est apparu d'importance dans nos conditions. En effet, le PFD sans propriétés antioxydantes maintient uniquement la viabilité des îlots. HEMOXCell<sup>®</sup> avait au contraire, eu un effet à la fois sur la viabilité et la fonction.

Il a été démontré que l'activité antioxydante avait une importance dans la détection du glucose ou l'entrée du glucose dans les cellules [251]. Pour confirmer que l'activité SOD est impliquée dans la maintenance de la fonction, l'inhibition intrinsèque SOD en utilisant du diéthyle dithiocarbamate fournira des informations sur l'impact de cette propriété spécifique sur les îlots.

La capacité d'oxygénation d'HEMOXCell<sup>®</sup> est corrélée à l'affinité de la protéine avec l'oxygène; Le P50 d'HEMOXCell<sup>®</sup> est à 37mmHg à 37°, ce qui veut dire que dans nos conditions drastiques de PO<sub>2</sub> (comme dans le dispositif), HEMOXCell<sup>®</sup> libère de l'oxygène. Mais il est possible que les conditions se dégradent liées à l'activité des îlots. En effet, pour sécréter de l'insuline, il est nécessaire d'avoir de l'ATP. La sécrétion d'insuline par stimulation au glucose dépend de l'activité des canaux du potassium sensibles à l'ATP (KATP), qui sont modulés par le ratio ATP/ADP, dépendant de la production d'ATP par la chaîne respiratoire mitochondriale par la phosphorylation oxydative [233]. Afin de générer l'ATP, la façon la plus rentable est le Cycle d'Acide Tricarboxylique qui requiert de l'oxygène comme accepteur d'électrons. En répondant à une stimulation au glucose, les îlots pourraient utiliser la moindre molécule d'O<sub>2</sub> pour utiliser le Cycle d'Acide Tricarboxylique, ils aggraveraient les conditions hypoxiques en faisant chuter transitoirement la concentration en oxygène en condition post-prandiale. Une fois tout l'oxygène utilisé, HEMOXCell<sup>®</sup> est déchargé et nécessite un apport en oxygène afin de le restocker.

Des études ont évalué l'efficacité d'HEMOXCell<sup>®</sup> à plus long terme, afin d'alimenter en oxygène les îlots. Après 4 jours sous hypoxie et sous confinement et en présence d'HEMOXCell<sup>®</sup> la masse d'îlot a diminué dramatiquement. Toutefois les îlots étaient toujours viables et avaient maintenu leur fonction. La raison de cette diminution de masse d'îlots pourrait être attribuée à l'épuisement des ressources en oxygène. Par conséquent le rechargement d'HEMOXCell<sup>®</sup> est un point critique.

Un des aspects intéressants de ce type d'hémoglobine marine est le panel d'hémoglobine avec les différents P50 pouvant potentiellement exister, dépendant de l'animal marin prélevé. Il y a différents types d'hémoglobine disponibles chez HEMARINA. Par exemple dans cette étude, nous avons testé HEMOXCell<sup>®</sup>, avec une P50 à 37mmHg à 37°C. En fait HEMARINA produit d'autres hémoglobines notamment HEMO2life<sup>®</sup> avec une P50 à 7mmHg. Cette hémoglobine spécifique présente un aspect intéressant car elle est utilisée dans le cadre d'essai cliniques pour la transplantation rénale. HEMO2life<sup>®</sup> a cependant un P50 plus bas qu'HEMOXCell<sup>®</sup>, qui correspond à une plus grande affinité à l'oxygène. La combinaison des deux créera comme un double système de déclenchement. HEMO2life<sup>®</sup> libérerait de l'oxygène pour recharger l'HEMOXCell<sup>®</sup> et serait intégré dans le système pour éviter l'anoxie complète (**Fig. 5-1**).



**Figure 5-1 Libération d'oxygène d'HEMOXCell<sup>®</sup> et HEMO2life<sup>®</sup>**

HEMOXCell<sup>®</sup> et tat-CREB ont pu maintenir la viabilité et la fonction des îlots pendant 1 jour, il serait intéressant de mélanger les deux molécules pour évaluer les effets sur les îlots; tout en gardant en mémoire que la combinaison de plusieurs molécules pourrait avoir des effets antinomiques. Comme prouvé par nos premières expériences en utilisant un mélange de PFD, d'adénosine et de tat-CREB, les résultats obtenus étaient moins bons que pour chaque molécule utilisée séparément.

Une fois que le maintien de la survie et de la fonction des îlots est garanti, il serait utile d'améliorer la sécrétion d'insuline pour diminuer les besoins des îlots dans le dispositif. Il a été démontré que GLP-1 analogue, le Liraglutide<sup>®</sup> favorise la réactivité des îlots en culture au glucose [252]. Ceci a été attribué à l'activité du récepteur GLP-1 au cAMP [252, 253].

Toutefois, le traitement avec Liraglutide<sup>®</sup>, pour des îlots sous confinement et en hypoxie pendant 24 heures était inefficace dans l'amélioration de la fonction des îlots, malgré la diminution en hypoxie en co-administrant du PFC et de l'adénosine. Nous avons encore à tester l'effet de la Liraglutide en combinaison avec HEMOXCell<sup>®</sup> et tat-CREB pour tester s'il est possible de maintenir la viabilité et la fonction des îlots et même booster la fonction sous de telles conditions drastiques de culture.

Pour finir sur le design du dispositif, le microenvironnement nécessite d'être amélioré. En effet, les matrices ont été extrêmement bénéfiques pour des îlots en culture [254]. De plus, l'intégration des îlots dans une matrice éviterait l'adhésion/fusion des îlots. Les matrices innovantes à base de matrices extracellulaires (faites de collagène, de laminine, de fibronectine, des facteurs de croissance, des glycominoglycans, des glycoprotéines et de proteoglycans) avec une viscosité appropriée pourraient être injectées avec les îlots pancréatiques dans le dispositif [255]. Certaines matrices peuvent même produire de l'oxygène. Pedraza *et al.* avait démontré que les biomatériaux sous forme de polydiméthylsiloxane (PDMS)-encapsulant du calcium peroxyde solide, PDMS-CaO<sub>2</sub> sont capables d'augmenter le PO<sub>2</sub> [240] en générant de l'oxygène. La présence d'un seul disque PDMS-CaO<sub>2</sub> diminue l'hypoxie de lignées cellulaires β et rétablit leur fonction. La PO<sub>2</sub> était autour de 110 mmHg pendant les premiers jours et a diminué progressivement pour atteindre 45 mmHg après 25 jours dans une atmosphère à 37 mmHg PO<sub>2</sub> [256, 240]. Toutefois, une fois que l'oxygène est diffusé, il n'est pas rechargeable. L'idée du pancréas bioartificiel est de créer un système autonome, qui permettrait d'espacer les interventions le plus possible, pour permettre au patient d'oublier la maladie pendant un moment. L'utilisation d'une matrice comme le PDMS-CaO<sub>2</sub>, combiné avec HEMOXCell<sup>®</sup> pourrait être une approche intéressante pour diffuser de l'oxygène plus longtemps comme une triple détente, le PDMS-CaO<sub>2</sub> qui pourrait recharger le HEMO2life<sup>®</sup> qui rechargerait le HEMOXCell<sup>®</sup>. De plus, la combinaison avec le tat-CREB, pourrait procurer l'environnement idéal pour les cellules sécrétrices d'insuline et permettre au dispositif d'encapsulation de fonctionner pendant longtemps sans aucune intervention. Une autre alternative serait d'utiliser l'hémoglobine matrice de base développée par HEMARINA diffusant de l'oxygène.

D'autres molécules font l'objet d'études, comme des matrices de protéines issues de la matrice extracellulaires, des agents antioxydants, et d'autres protéines, et ont permis d'obtenir des candidats prometteurs qui pourraient être mélangés avec HEMOXCell<sup>®</sup> et tat-CREB dans un nouveau milieu réunissant tous les besoins pour la survie de cellules à usage médicale. C'est l'un des objectifs du projet BIOSID.

Le Projet de support spécifique dédié aux cellules encapsulées va préserver la viabilité pendant un moment, mais le but n'est pas d'obtenir des cellules immortelles, qui en terme d'éthique serait problématique. Prenant en compte ces paramètres, le MAILPAN<sup>®</sup> a été conçu pour permettre de remplir le dispositif avec des cellules. C'est l'avantage majeur de ce dispositif par rapport aux autres. Le principe est de remplir le dispositif avec des cellules quelques semaines après l'implantation, et les laisser fonctionner pendant un laps de temps et une fois que la glycémie n'est plus régulée, procéder à la vidange et au remplacement avec de nouvelles cellules. Comme le nombre d'îlots qui reversent le diabète est très élevé, et comme le remplacement peut se produire trimestriellement, l'utilisation d'îlots humains est inconcevable pour cette application car il y a un manque de dons d'îlots humains, un manque de donneur, ainsi qu'un faible rendement de tout le processus d'isolation.

Par conséquent, la prochaine étape sera l'utilisation de cellules de substitution. Certaines lignées de cellules souches pourraient présenter une résistance à l'hypoxie [257], qui combinée avec le milieu étudié spécifiquement pour les cellules encapsulées utilisant HEMOXCell® et tat-CREB pourraient assurer la fonction sur une durée plus longue pour le pancréas bio artificiel.

Les îlots xénogéniques et les îlots porcins en particulier, sont considérés comme une alternative potentielle. Cependant, la préoccupation majeure dans la xénotransplantation est xénozoonose, qui est le risque de développer de nouvelles infections se transmettant de l'animal à l'homme. La peur de la transmission rétrovirus endogènes (PERV) de porc a stoppé les essais cliniques pour près d'une décennie. Tous les modèles porcins présentent des PERV, ils ont subi plusieurs mutations au fil du temps dans le génome du porc et sont transmis à la descendance. La plupart des races de porcs contiennent 50 génomes de PERV dans leur ADN, et même si ils ne provoquent pas l'infection chez le porc, il a le potentiel de provoquer une infection chez d'autres espèces [116, 117]. Cependant, il n'a jamais été montré de transmission à l'homme ou le singe après transplantation de tissu porcine [134, 258]. Paradis *et al.* a effectué une étude rétrospective sur des sérums et des cellules de 14 patients diabétiques traités avec des îlots pancréatiques porcins. Malgré la présence de peptide C porcine dans les urines de quatre patients 257 et 240 jours après la xéno greffe, cependant, aucune preuve d'infection active n'a été détectée en testant dans le sérum ou la salive [259]. À ce jour, il n'y a eu aucune indication d'infections chez les petits animaux ou des primates non humains.

Les études réalisées par Dufrane *et al.* ont démontré que les îlots de porc micro encapsulés dans l'alginate étaient fonctionnels durant 6 mois après l'implantation dans un modèle xénogénique sans immunosuppression. 15,000 microcapsules IEQ/kg ont été introduites dans les reins sous capsulaires, l'organe receveur des primates, car la pression locale d'oxygène y est élevée et la récupération des capsules est facilitée pour l'analyse. 135 à 180 jours après l'implantation, certains îlots ont survécus et étaient capables de réagir *in vitro* à une stimulation en glucose [236].

Néanmoins, les îlots de porc sont reconnus pour avoir une libération d'insuline faible ce qui pourrait être un problème pour l'utilisation d'îlots de porc dans la transplantation [148]. Afin d'améliorer la fonction des îlots de porc, on a réalisé une étude sur la co-culture d'îlots de porc avec des cellules souches mésenchymateuses (MSCs). L'utilisation de MSCs a amélioré la vascularisation de la greffe dans un modèle de co-transplantation d'îlots de rein dérivés MSCs [260]. L'encapsulation d'îlots de porc et des MSCs améliore la vascularisation dans la périphérie d'îlots co-encapsulés en corrélation avec une teneur plus élevée en oxygène de la greffe [147]. Toutefois, dans nos conditions, les MSCs n'étaient pas efficaces voir toxiques pour la fonction et la viabilité des îlots car le composite créé par MSC recouvrant les îlots avait créé plus d'hypoxie (données non communiquées).

L'utilisation des îlots de porc dans les dispositifs macro encapsulés n'a jamais été démontré comme fonctionnant car ces derniers sont réellement fragiles et sensibles à l'encapsulation. Il serait intéressant de tester HEMOXCell® et tat-CREB sur ces îlots pour voir si le maintien de la fonction peut être réalisé.

Différents essais ont été réalisés ces 30 dernières années afin de générer des cellules humaines  $\beta$  avec plusieurs provenances pancréatiques, comme les îlots adultes, le pancréas fœtal, ou insulinome. Toutefois la production d'insuline produite par ces cellules était extrêmement faible ou alors ces cellules n'étaient capables de produire de l'insuline que sur quelques



passages [261, 262]. Récemment, plusieurs lignées cellulaires se sont démarquées. Melton et al [s14] ont examiné plusieurs façons de créer des cellules  $\beta$  (SC- $\beta$  cells) à partir de hPSC. Ils ont testés plusieurs combinaisons d'étapes séquentielles de culture utilisant des facteurs qui influent les signaux dans plusieurs voies. Pour tester leur capacité à fonctionner *in vivo*, 5 million de cellules SC- $\beta$  ont été transplantées sous la capsule rénale chez des souris immunodéprimées. Des mesures d'insulines réalisées dans le sérum deux semaines après transplantation ont montré que les cellules SC- $\beta$  sécrètent de l'insuline. Après 2 semaines de post-transplantation, les animaux ont été sacrifiés et les reins greffés ont montrés que les cellules greffées SC- $\beta$  contenaient des cellules C-peptides positives adjacentes aux reins de la souris [263]. Scharfmann *et al.* ont développé une lignée cellulaire issue de tissus pancréatique fœtal après deux modifications génétiques successives. Premièrement, le tissu fœtal a été transfecté en utilisant un vecteur lentiviral exprimant SV40LT mené par le promoteur de l'insuline comme il a été utilisé pour le développement de lignées cellulaires  $\beta$  de rongeurs. La deuxième étape consiste en une deuxième transfection avec la télomérase transcriptase inverse humaine (hTERT) permettant l'immortalisation des cellules. La génération d'EndoC- $\beta$ H1 était une énorme découverte dans le domaine car ce fut la première fois qu'une lignée cellulaire humaine fonctionnelle était générée. Effectivement, ces cellules contenaient de l'insuline et étaient capables de répondre à la stimulation au glucose et le plus important de reverser le diabète chez les souris [264]. Cette découverte mène à la la création d'Endocell, une société en charge du développement et de l'amélioration de la lignée cellulaire. Depuis 2 générations de cellules EndoC- $\beta$ H ont été développées, EndoC- $\beta$ H2 et plus récemment EndoC- $\beta$ H3 pour lesquels le but est plus la transplantation avec la problématique de stopper la réplication. Dans le projet BIOSID, ces cellules vont être testées dans le MAILPAN<sup>®</sup>. sur un modèle de rongeur diabétique Cela serait une alternative intéressante car elles semblent plus résistantes à l'hypoxie que les îlots et pourraient être combinées avec HEMOXCell<sup>®</sup> et tat-CREB afin de maintenir leur survie à long terme.

En conclusion, tout ce travail sera utile dans le domaine de l'encapsulation mais également, connaissant les besoins des îlots, dans le design de nouveaux supports.

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## PUBLICATIONS

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**A. Rodriguez-Brotons**, W. Bietiger, C. Peronet, J. Magisson, C. Sookhareea, A. Langlois, C. Mura, N. Jeandidier, M. Pinget, S. Sigrist, E. Maillard. Impact of pancreatic rat density on cell survival during hypoxia. *Journal of Diabetes Research* 2016

**A. Rodriguez-Brotons**, W. Bietiger, C. Peronet, J. Magisson, C. Mura, C. Sookhareea, N. Jeandidier, V. Polard, F. Zal, M. Pinget, S. Sigrist, E. Maillard. Comparison between perfluorodecalin and HEMOXCell<sup>®</sup> as oxygen carrier for islet encapsulation (Submitted)

**A. Rodriguez-Brotons**, J. Magisson, W. Bietiger, C. Peronet, A. Sassi, C. Sookhareea, C. Mura, N. Jeandidier, M. Pinget, S. Sigrist, E. Maillard. Improvement of inflammation in a confined and hypoxic environment (In preparation)

## ORAL COMMUNICATIONS

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**A. Rodriguez-Brotons**, H. Hamdard, W. Bietiger, M. Pinget, N. Jeandidier, E. Maillard, S. Sigrist. Preliminary study on influence of confinement on rat islet survival. AIDPIT Workshop 2014, Innsbruck, Austria.

## POSTERS

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**A. Rodriguez-Brotons**, C. Mura, W. Bietiger, C. Peronet, E. Seyfritz, C. Sookhareea, S. Sigrist, E. Maillard. Besoin et contraintes du pancréas bioartificiel. Société francophone de transplantation, SFT, 2014, Caen, France.

**A. Rodriguez-Brotons**, E. Maillard, S. Sigrist. Improvement of islet survival in a hypoxic and confined environment by oxygenated adenosine and perfluorodecalin solution. AIDPIT Workshop 2015, Innsbruck, Austria.

**A. Rodriguez-Brotons**, C. Mura, W. Bietiger, C. Peronet, E. Seyfritz, S. Sigrist, E. Maillard. Pancreatic islet survival under confinement. American Transplantation Congress, ATC 2015, Philadelphia, USA.

**A. Rodriguez-Brotons**, C. Mura, W. Bietiger, C. Peronet, E. Seyfritz, S. Sigrist, E. Maillard. Bioartificial pancreas, new strategy to treat type 1 diabetes. Advanced technologies and treatments for diabetes, ATTD 2015, Paris, France.

**A. Rodriguez-Brotons**, E. Maillard, S. Sigrist, S. Dalle. Restoration of islet survival and inflammation decrease in a hypoxic and confined environment. AIDPIT Workshop 2016, Innsbruck, Austria.



# Aida Rodriguez-Brotons

## Amélioration de la viabilité des îlots pancréatiques dans le pancréas bioartificiel

### **Amélioration de la viabilité des îlots pancréatiques dans le pancréas bioartificiel**

La transplantation d'îlots pancréatiques est considérée comme une thérapie prometteuse quant au traitement du diabète de type 1. En revanche, l'utilisation de traitements immuno-suppresseurs ainsi que le manque de donneur sont un frein à l'expansion de cette thérapie à plus de patients diabétiques. Pour résoudre ces deux problèmes, la stratégie développée durant ces vingt dernières années est le pancréas bioartificiel. Il consiste en une immuno-isolation de la greffe dans une enveloppe artificielle, protégeant non seulement la greffe du système immunitaire, mais aussi le receveur de la greffe. Les îlots ou les cellules productrices d'insuline transplantée(s) ne sont pas en contact avec le système immunitaire et aucune immunosuppression n'est nécessaire. L'objectif de ce travail était de déterminer les marqueurs de survie et de mort des îlots dans des conditions mimant celles du MAILPAN<sup>®</sup>, un pancréas bioartificiel développé par Defymed et d'étudier différentes molécules qui pourraient augmenter la survie des îlots. Nous avons démontré que cet environnement bioartificiel engendrait un confinement et de l'hypoxie créant un stress cellulaire et donc une perte précoce des îlots. Nous avons identifié plusieurs cibles pour améliorer la viabilité et la fonction comme par exemple les transporteurs d'oxygène ou des molécules anti-inflammatoires. Plus le nombre d'îlots dans le MAILPAN<sup>®</sup> était élevé, plus les effets délétères sur la survie des îlots étaient importants. En conséquence, nous avons testé différentes molécules impliquées dans les voies de l'hypoxie et de l'inflammation pour augmenter la survie et restaurer la fonction des îlots pancréatiques dans un environnement confiné et hypoxique (600IEQ/cm<sup>2</sup>). L'ajout d'HEMOXcell<sup>®</sup>, un nouveau transporteur d'oxygène et du peptide tat-CREB ont montré une restauration de la fonction des îlots ainsi qu'une diminution de l'hypoxie et de l'inflammation après 24h de culture. Ainsi ce travail a permis l'identification de nouveaux candidats pour l'élaboration d'un milieu spécialisé pour l'encapsulation de cellules.

### **Improvement of pancreatic islets viability in the bioartificial pancreas**

Islet transplantation is considered as promising therapy for brittle type 1 diabetes. However, the use of immunosuppressive regimen and the lack of donor prevent the expansion of the therapy to other diabetic patients. In order to address these two issues, the strategy developed for the two last decades is the bioartificial pancreas. It consists in the immune-isolation of the graft in an artificial envelop, protecting at the same time the graft, from the immune-system, and the host, from the graft. In principle, the transplanted islets or surrogate insulin secreting cells are not in contact with the immune system and no immunosuppressive drugs are needed. The objective of this work was to identify the markers of islet death/survival mimicking MAILPAN<sup>®</sup> conditions, a bioartificial pancreas developed by Defymed and study different molecules which can improve islet survival. We demonstrated that bioartificial environment induced confinement and hypoxia which triggers cellular distress causing early islet loss. We identified several targets to improve viability and function such as oxygen carriers or anti-inflammatory drugs. The highest the number of islets in the MAILPAN<sup>®</sup> was, the most deleterious effects in islet survival and functionality were observed. As a consequence, we tested different molecules implicated in hypoxia and inflammation pathway to improve islet survival and restore islet functionality in a hypoxic and confined environment (600IEQ/cm<sup>2</sup>). The addition of HEMOXCell<sup>®</sup>, a novel oxygen carrier, and tat-CREB peptide have been shown to restore islets functionality and decrease hypoxia and inflammation levels after 24 hours in culture. Thus, these data provide new inputs for the design of a culture medium dedicated for cell encapsulation.