REGENERATION AND PROTECTION OF BETA CELLS IN TYPE 1 DIABETES

Thesis leading to the title of
International PhD in Endocrinology and Metabolic Diseases

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To Cecilia
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INDEX

LIST OF FIGURES

	pancreas of NOD mice feeded with diet RM3	
Figure 42	Reg3gamma gene expression in the pancreas of NOD mice feeded with diet RM1	Personal data elaboration
Figure 43	Reg3gamma gene expression in the pancreas of NOD mice feeded with diet RM3	Personal data elaboration
Figure 44	Reg3delta gene expression in the pancreas of NOD mice feeded with diet RM1	Personal data elaboration
Figure 45	Reg3delta gene expression in the pancreas of NOD mice feeded with diet RM3	Personal data elaboration

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ABSTRACT (English version)

T1D is an immune-mediated disease leading to the destruction of insulin producing cells and its frequency is increasing worldwide. As a result, islet cells are destroyed and, in the absence of insulin, glucose cannot enter the cells and accumulates in the blood. Replacement of the insulin producing cells (beta cells) represents the ultimate treatment for T1D. Recent advances in islet transplantation underscore the urgent need for developing alternatives to human tissue donors, which are scarce. Two possible approaches are the generation of insulin producing cells from embryonic or adult stem cells and the regeneration of endogenous beta cells.

The overall aim of my PhD was to find out which of these approaches could be more feasible and which one could give the best results.

The specific aim of the first part of this PhD was to study the best conditions for the isolation and culture of mesenchymal stem cells (MSCs) obtained from the bone marrow of non-obese diabetic (NOD) mice. MSCs represent one of the ideal sources that can be used for the generation of new insulin producing cells. The rationale behind this choice of experiment was that these cells will provide an unlimited source of autologous stem cells which can be transformed into insulin producing cells.

At the same time we analyzed the potentiality of regeneration of the beta cells. This is a new and promising area of the research and it is important to determine its feasibility. It is therefore mandatory to arrange a marker that can be easly use to determine beta cell regeneration and following this

line we set up our experiments on Reg genes and regeneration in NOD mice and also in human samples.

Finally, during this PhD, I had the opportunity to meet Professor Terence Wilkin, Professor of Endocrinology & Metabolism at the University Medicine of Plymouth (UK), and together we set up a project on the Accelerator Hypothesis in the NOD mouse.

This is a research project aimed to investigate mice exposed to different food availability, food restriction compared to the normal situation in which mice have free access to food (ad libitum), and how the age at onset of diabetes varied accordingly. Culled mice at diabetes diagnosis were dissected and pancreas retained for studies aimed at expression of Reg genes (Reg1, Reg2, Reg 3a, Reg3ß, Reg 3?, and Reg 3d) associated with beta cell regeneration.

Furthermore, with the present study, we tested the hypothesis that T1D can be delayed/prevented in the NOD mouse, by limiting weight gain (Accelerator Hypothesis) and therefore reducing the stress to the beta cells.

Finally we tested the hypothesis that a diet with a lower protein and a greater starch amount could also impact disease development and beta cell regeneration.

ABSTRACT (Italian version)

Il diabete di tipo 1 (T1D) è una malattia immuno mediata che conduce alla completa perdita delle cellule che secernono insulina (beta cellule) e la sua frequenza è in continuo aumento. In assenza di insulina, il glucosio non può entrare nelle cellule e si accumula nel sangue. Il rimpiazzo di queste beta cellule rappresenta dunque l'unica possibilità per curare questa malattia.

I recenti progressi nel settore dei trapianti di isole pancreatiche hanno mascherato l'urgente bisogno di una cura alternativa in quanto il problema della disponibilità di organi da trapiantare è molto ampio. Due alternative possibili prevedono la generazione di nuove cellule secernenti insulina a partire da cellule staminali (embrionali o adulte) e la generazione di nuove beta cellule endogene.

Lo scopo principale di questo PhD è stato quello di andare a studiare quale di questi due approcci può essere più fattibile e quale dei due può offrire i migliori risultati.

Scopo specifico della prima parte di questo PhD è stato di approfondire la metodica per isolare e coltivare cellule staminali mesenchimali isolate dal midollo osseo del topo NOD.

A mio avviso queste cellule rappresentano la fonte ideale di cellule per generare nuove cellule secernenti insulina. Il razionale di questa scelta si fonda sul fatto che queste cellule mesenchimali possono fornire una fonte illimitata di cellule staminali autologhe.

Allo stesso tempo però mi sono dedicata anche allo studio del potenziale rigenerativo delle beta cellule. Questa è un nuovo settore della ricerca ed è importante studiarne la potenzialità. Lo scopo dello studio è stato quello di valutare la possibilità di disporre di un marker della rigenerazione beta cellulare e seguendo questo scopo ho impostato lo studio sui geni Reg (Reg1, Reg2, Reg3a, Reg3ß, Reg3? e Reg3d) e sulla rigenerazione sia nel modello animale (topo NOD) che in campioni umani.

Infine, durante il mio corso di dottorato, ho avuto la possibilità di incontrare il Professor T. Wilkin e di lavorare con lui per mettere a punto uno studio per testare l'Accelerator Hypothesis. Lo scopo del nostro studio è stato di valutare quali possano essere gli effetti una riduzione della quantità di cibo (4 gr/die) rispetto ad una normale dieta "ad libitum" sull'insorgenza del diabete nel modello animale del topo NOD. La relazione tra peso corporeo e insorgenza della malattia è stata esaminata facendo riferimento al peso alla nascita, alle variazioni di peso durante lo sviluppo e al peso corporeo al momento dell'insorgenza del diabete. In questi animali ho anche valutato l'espressione dei geni Reg correlati appunto alla rigenerazione beta cellulare. In questo modo dunque ho valutato l'ipotesi che il T1D può essere prevenuto/ritardato nel topo NOD limitando l'aumento del loro peso corporeo e riducendo lo stress sulle beta cellule.

CHAPTER 1: DIABETES MELLITUS

1.1 DEFINITION OF DIABETES MELLITUS

Diabetes mellitus is one of the most common endocrine disorders affecting almost 6% of the world's population. The number of diabetic patients will reach 300 million in 2025 (International Diabetes Federation, 2001).

The term ***diabetes mellitus*** describes a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. The effects of diabetes mellitus include long term damage, dysfunction and failure of various organs.

Diabetes mellitus may present with characteristic symptoms such as thirst, polyuria, blurring of vision and weight loss. In its most severe forms, ketoacidosis or a non ketotic hyperosmolar state may develop and lead to stupor, coma and in absence of effective treatment, death. Often symptoms are not severe, or may be absent, and consequently hyperglycaemia sufficient to cause pathological and functional changes may be present for a long time before the diagnosis is made.

The long term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and neuropathy with risk of foot ulcers, amputation, Charcot joints and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease.

Several pathogenetic processes are involved in the development of diabetes. These include processes which destroy the beta cells of the pancreas with consequent insulin deficiency, and others that result in resistance to insulin action. The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin.

1.2 CLASSIFICATION

In June 1997, an international expert committee released a report with new recommendations for the classification and diagnosis of diabetes mellitus[1]. These new recommendations were the result of more than two years of collaboration among experts from the American Diabetes Association and the World Health Organization (WHO). The use of classification systems and standardized diagnostic criteria facilitates a common language among patients, physicians, other health care professionals and scientists.

- **Previous Classification**

In 1979, the National Diabetes Data Group produced a consensus document standardizing the nomenclature and definitions for diabetes mellitus[2]. This document was endorsed one year later by WHO[3,4]. The two major types of diabetes mellitus were given names descriptive of their clinical presentation: "insulin-dependent diabetes mellitus" (IDDM) and "noninsulin-dependent diabetes mellitus" (NIDDM). However, as

treatment recommendations evolved, correct classification of the type of diabetes mellitus became confusing. For example, it was difficult to correctly classify persons with NIDDM who were being treated with insulin. This confusion led to the incorrect classification of a large number of patients with diabetes mellitus, complicating epidemiologic evaluation and clinical management. The discovery of other types of diabetes with specific pathophysiology that did not fit into this classification system further complicated the situation. These difficulties, along with new insights into the mechanisms of diabetes mellitus, provided a major impetus for the development of a new classification system.

The National Diabetes Data Group also established the oral glucose tolerance test (using a glucose load of 75 g) as the preferred diagnostic test for diabetes mellitus.[3] However, this test has poor reproducibility, lacks physiologic relevance and is a weaker indicator of long-term complications compared with other measures of hyperglycaemia[5]. Furthermore, many high-risk patients are unwilling to undergo this time-consuming test on a repeat basis. The new diagnostic criteria also address this issue.

- **Changes in the Classification System**

The new classification system identifies four types of diabetes mellitus: type 1, type 2, "other specific types" and gestational diabetes. Each of the types of diabetes mellitus identified extends across a clinical continuum of hyperglycaemia and insulin requirements.

T1D (formerly called type I, IDDM or juvenile diabetes) is characterized by beta cell destruction caused by an autoimmune process, usually leading to absolute insulin deficiency[6]. The onset is usually acute, developing over a period of a few days to weeks. Over 95 percent of persons with T1D develop the disease before the age of 25, with an equal incidence in both sexes and an increased prevalence in the white population. A family history of T1D, gluten enteropathy (celiac disease) or other endocrine disease is often found. Most of these patients have the "immune-mediated form" of T1D with islet cell antibodies and often have other autoimmune disorders such as Hashimoto's thyroiditis, Addison's disease, vitiligo or pernicious anemia. A few patients, usually those of African or Asian origin, have no antibodies but have a similar clinical presentation; consequently, they are included in this classification and their disease is called the "idiopathic form" of T1D.

T2D (formerly called NIDDM, type II or adult-onset) is characterized by insulin resistance in peripheral tissue and an insulin secretory defect of the beta cell. This is the most common form of diabetes mellitus and is highly associated with a family history of diabetes, older age, obesity and lack of exercise. It is more common in women, especially women with a history of gestational diabetes, and in blacks, Hispanics and Native Americans. Insulin resistance and hyperinsulinemia eventually lead to impaired glucose tolerance. Defective beta cells become exhausted, further fueling the cycle of glucose intolerance and hyperglycaemia. The aetiology of T2D

is multifactorial and probably genetically based, but it also has strong behavioral components.

Types of diabetes mellitus of various known etiologies are grouped together to form the classification called "other specific types." This group includes persons with genetic defects of beta cell function (this type of diabetes was formerly called MODY or maturity-onset diabetes in youth) or with defects of insulin action; persons with diseases of the exocrine pancreas, such as pancreatitis or cystic fibrosis; persons with dysfunction associated with other endocrinopathies (e.g., acromegaly); and persons with pancreatic dysfunction caused by drugs, chemicals or infections.

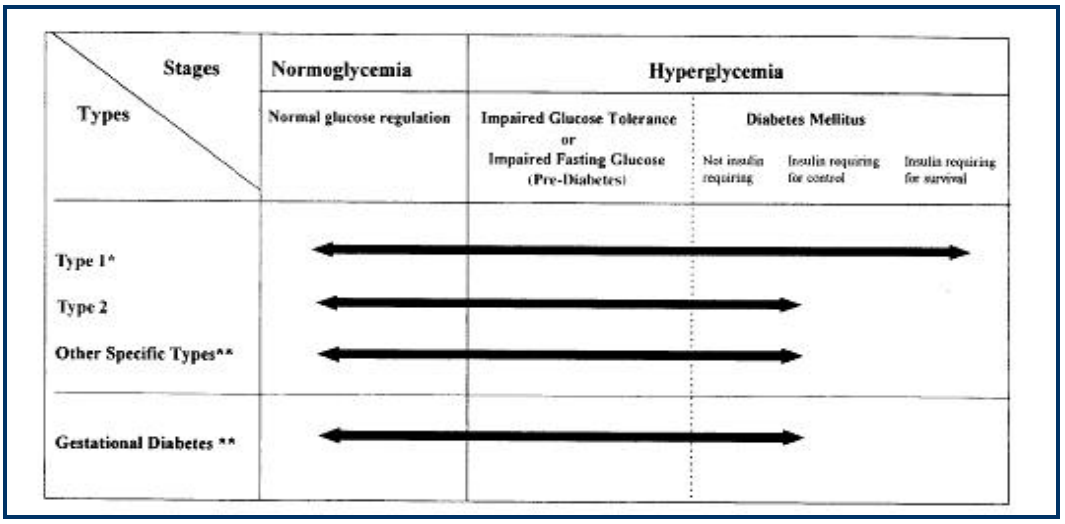


Figure 1. Disorders of glycaemia: etiologic types and stages

- **New classification**

In 1997, the American Diabetes Association (ADA) published new criteria for the diagnosis of diabetes[7]. They were introduced to facilitate wider

recognition of diabetes and to minimize the need for oral glucose tolerance testing to identify people with undiagnosed asymptomatic diabetes. The diagnostic level for fasting plasma glucose (FPG) was set at =7.0 mmol/l to minimize the discrepancy in the 1985 WHO criteria, by which diabetes was diagnosed by either FPG =7.8 mmol/l or 2-h postload plasma glucose (2-h PG) =11.1 mmol/l during a 75-g oral glucose tolerance test (OGTT) [8].

In participants in the second National Health and Nutrition Examination Survey, only 23% of those with newly diagnosed diabetes by the 1985 WHO criteria had FPG =7.8 mmol/l, whereas 97% had 2-h PG =11.1 mmol/l[9]. Thus, most people being tested for diabetes would not be diagnosed without an OGTT, a procedure not routinely performed in clinical practice unless diabetes is suspected.

The ADA criteria are based primarily on FPG, which, if =7.0 mmol/l, is provisionally diagnostic of diabetes. A clinical diagnosis requires confirmation on repeat testing. Although the ADA recommendations do allow for diagnosis by OGTT (if the 2-h PG is =11.1 mmol/l) or by high casual plasma glucose in the presence of symptoms, the ADA recommends using only the fasting level with the FPG criterion of FPG =7.0 mmol/l for determining the prevalence or incidence of diabetes.

The prevalence of undiagnosed diabetes by ADA criteria is lower than by the 1985 WHO criteria, but implementation of the ADA recommendations in clinical practice and screening will likely result in a more complete discovery of people with undiagnosed diabetes and detection at an earlier stage. Yet, there is concern that the diagnosis of diabetes by FPG alone

using the ADA criteria will fail to identify people who would be diagnosed by glucose tolerance testing using the 1985 WHO criteria. In 1999, the WHO made further recommendations regarding criteria for diagnosis of diabetes and other categories of impaired glucose regulation[10]. They incorporate the change in the FPG diagnostic level to =7.0 mmol/l but retain the recommendation for the OGTT and diagnosis of diabetes if the 2-h PG is =11.1 mmol/l.

1.3 EPIDEMIOLOGY OF TYPE 1DIABETES

The incidence rate of T1D varies with age and rarely occurs before the first 6 months of age[11] with the exception of a very few cases recorded in a large registry in the UK[12]. The incidence of T1D begins sharply to rise at about 9 months of age, continues to rise until age 12– 14 years, and then declines[13]. A similar pattern is seen in many other countries irrespective of whether the overall incidence of T1D is low or high[14].

Available data in the USA suggest that there is a temporal increase in the incidence of T1D over the last 50 years from about 5% in 1940 to 20% in 1980[15].

Many studies have been performed on the incidence rate of diabetes in various parts of the world to allow for examination of the geographical pattern of T1D. The incidence of T1D is lowest in Japan, the Caribbean, and southern Europe while the highest incidence rates are in the Scandinavian countries[16], particularly in Finland, and in Sardinia in Italy[17]. The incidence rate of T1D in the white population of the USA is higher

than those recorded for countries of northern Europe but significantly lower than those in Sweden and Finland. The incidence of T1D in African Americans was lower than in white Americans [18]. Accumulated data on the incidence of T1D during the last 20 years show that T1D occurs in most racial and ethnic groups but the risk is highest among white population. All of these examples support the role of genetic and environmental factors in the aetiology of diabetes. A more recent study shows that also climate has a role in the aetiology of T1D[19]. The continued increase in the incidence of T1D in many parts of the world would suggest that the role of environmental factors in the aetiology of diabetes mellitus is extremely important[20].

1.4 EPIDEMIOLOGY OF TYPE 2 DIABETES

The overall prevalence of T2D in USA population aged 20–74 years was 6.6% in 1980, corresponding to more than 8 million people. The prevalence of T2D was slightly higher in women than in men, except for the age group 65–74 years. The prevalence of T2D among blacks was higher than among whites at all ages and for both sexes[21]. The prevalence in Hispanic minorities was even higher[22] but did not approach that of Pima Indians of Arizona[23].

The prevalence of T2D in the European population is relatively low compared with the prevalence recorded for these American populations. In European populations, the prevalence of T2D is less than half of the prevalence observed in American populations[24]. The prevalence of T2D in Saudi Arabia is similar to that obtained in USA populations, while in

Central Asia the prevalence among men aged 50 years and over is similar to that in European men[25].

The substantial difference in the occurrence of diabetes among white populations, particularly between those in Europe and North America, points to an environmental component in the development of diabetes mellitus in white populations.

1.5 DIAGNOSIS OF DIABETES

The classical triad of diabetes symptoms is polyuria, polydipsia and polyphagia. Symptoms may develop quite rapidly (weeks or months) in T1D, particularly in children. However, in T2D the symptoms develop much more slowly and may be subtle or completely absent. T1D may also cause a rapid yet significant weight loss (despite normal or even increased eating) and irreducible fatigue. All of these symptoms except weight loss can also manifest in T2D in patients whose diabetes is poorly controlled.

When the glucose concentration in the blood is raised beyond the renal threshold, reabsorption of glucose in the proximal renal tubuli is incomplete and part of the glucose remains in the urine (glycosuria). This increases the osmotic pressure of the urine and inhibits the reabsorption of water by the kidney, resulting in increased urine production (polyuria) and increased fluid loss. Lost blood volume will be replaced osmotically from water held in body cells, causing dehydration and increased thirst.

Prolonged high blood glucose causes glucose absorption, which leads to changes in the shape of the lenses of the eyes, resulting in vision changes. Blurred vision is a common complaint leading to a diabetes diagnosis; T1D

should always be suspected in cases of rapid vision change whereas T2D is generally more gradual, but should still be suspected.

Patients, usually with T1D, may also present with diabetic ketoacidosis (DKA), an extreme state of metabolic dysregulation characterized by the smell of acetone on the patient's breath; a rapid, deep breathing known as Kussmaul breathing; polyuria; nausea; vomiting and abdominal pain; and any of many altered states of consciousness or arousal. In severe DKA, coma may follow, progressing to death. A rarer but equally severe possibility is hyperosmolar nonketotic state, which is more common in T2D and is mainly the result of dehydration due to loss of body water. Often, the patient has been drinking extreme amounts of sugar-containing drinks, leading to a vicious circle in regard to the water loss.

The following criteria are used to make diagnosis of diabetes:

- Symptoms of diabetes plus casual plasma glucose concentration = 200 mg/dl (11.1 mmol/l). Casual is defined as any time of day without regard to time since last meal.
- Fasting Plasma Gluocse (FPG) = 126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.
- 2-h postload glucose = 200 mg/dl (11.1 mmol/l) during an oral glucose tolerance test (OGTT). The test should be performed as described by WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

Diabetes mellitus--positive findings from any two of the following tests on different days:

Symptoms of diabetes mellitus* plus casual† plasma glucose concentration >=200 mg per dL (11.1 mmol per L)
or
FPG >=126 mg per dL (7.0 mmol per L)
or
2hrPPG >=200 mg per dL (11.1 mmol per L) after a 75-g glucose load

Impaired glucose homeostasis

Impaired fasting glucose: FPG from 110 to <126 (6.1 to 7.0 mmol per L)
Impaired glucose tolerance: 2hrPPG from 140 to <200 (7.75 to <11.1 mmol per L)

Normal

FPG <110 mg per dL (6.1 mmol per L)
2hrPPG <140 mg per dL (7.75 mmol per L)

Adapted from Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 1997; 20:1183-97.

†--Casual is defined as any time of day without regard to time since last meal.

*--Symptoms include polyuria, polydipsia or unexplained weight loss.

FPG=fasting plasma glucose; 2hrPPG=two-hour postprandial glucose.

Figure 2. Criteria for the Diagnosis of Diabetes Mellitus and Impaired Glucose Homeostasis.

1.6 FEATURES OF TYPE 1 DIABETES

This form of diabetes, previously called insulin–dependent diabetes or juvenile onset diabetes, results from autoimmune mediated destruction of the beta cells of the pancreas. The rate of destruction is quite variable, being rapid in some individuals and slow in others[26].The rapidly

progressive form is commonly observed in children, but also may occur in adults[27]. The slowly progressive form generally occurs in adults and is sometimes referred to as latent autoimmune diabetes in adults (LADA). Some patients, particularly children and adolescents, may present with ketoacidosis as the first manifestation of the disease[28]. Others have modest fasting hyperglycaemia that can rapidly change to severe hyperglycaemia and/or ketoacidosis in the presence of infection or other stress. Still others, particularly adults, may retain residual beta cell function, sufficient to prevent ketoacidosis, for many years [29].

Individuals with this form of T1D often become dependent on insulin for survival eventually and are at risk for ketoacidosis [30]. At this stage of the disease, there is little or no insulin secretion as manifested by low or undetectable levels of plasma C–peptide[31]. Markers of immune destruction, including thyrosine phosphatise autoantibodies (IA-2), autoantibodies to insulin (IAA) and autoantibodies to glutamic acid decarboxylase (GAD) are present in 85–90 % of individuals with T1D when fasting diabetic hyperglycaemia is initially detected[32]. The peak incidence of this form of T1D occurs in childhood and adolescence, but the onset may occur at any age, ranging from childhood to the ninth decade of life[33]. There is a genetic predisposition to autoimmune destruction of beta cells, and it is also related to environmental factors.

Age and season at diagnosis of T1D influence the amount of residual beta cell function, which is higher in subjects with the onset of the disease in adult age as compared to those diagnosed in childhood or in adolescence[34,35].

Furthermore, we have shown that a lack of correlation between HbA1c and C-peptide values (with the lowest C-peptide levels found in subjects < 9 years of age at diagnosis) indicates that the process of beta cell damage is very destructive and unique in this young age group, probably reflecting a different pathogenic disease process.

1.7 FEATURES OF TYPE 2 DIABETES

Diabetes mellitus of this type was previously called non insulin dependent diabetes or adult onset diabetes. It is a term used for individuals who have a relative, rather than absolute, insulin deficiency. People with this type of diabetes frequently are resistant to the action of insulin[36,37]. At least initially, and often throughout their lifetime, these individuals do not need insulin treatment to survive. This form of diabetes is frequently undiagnosed for many years because the hyperglycaemia is often not severe enough to provoke noticeable symptoms of diabetes [38]. Nevertheless, such patients are at increased risk of developing macrovascular and microvascular complications[39]. Although the specific aetiologies of this form of diabetes are not known, by definition autoimmune destruction of the pancreas does not occur and patients do not have other known specific causes of diabetes.

The majority of patients with this form of diabetes are obese and obesity itself causes or aggravates insulin resistance[40,41].Many of those who are not obese by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region[42]. Ketoacidosis is infrequent in this type of diabetes; when seen it usually arises in association with the stress of another illness such as infection[43,44]. Whereas patients with this form of diabetes may have insulin levels that appear normal or elevated, the high blood glucose levels in these diabetic patients would be expected to result in even higher insulin values had their beta cell function been normal[45]. Thus, insulin secretion is defective and

insufficient to compensate for the insulin resistance. On the other hand, some individuals have essentially normal insulin action, but markedly impaired insulin secretion. Insulin sensitivity may be increased by weight reduction, increased physical activity, and/or pharmacological treatment of hyperglycaemia but is not restored to normal[46,47]. The risk of developing T2D increases with age, obesity and lack of physical activity[48]. It occurs more frequently in women with prior GDM and in individuals with hypertension or dyslipidaemia. Its frequency varies in different racial/ethnic subgroups[49] and it is often associated with strong familial, likely genetic, predisposition[50,51,52]. However, the genetic of this form of diabetes is complex and not clearly defined.

Among patients diagnosed with T2D some of them develop insulin-requiring diabetes during follow-up and some of these patients can be identified earlier in the natural history of the disease by the presence of circulating islet autoantibodies[53]. This form of diabetes also referred to as latent autoimmune diabetes in adults (LADA), according to the latest classification, is now considered a form of T1D [54,55].

Several studies have been performed to evaluate the prevalence of LADA in patients with adult-onset diabetes based on screening for ICA and/or GAD. Some of these studies also evaluated the correlation between the presence of GAD and the clinical features of affected patients. A key question is whether the process of beta cell destruction follows the same pattern in patients diagnosed in young age, after adolescence, or in adult age. The speed of beta cell loss probably varies according to genetic, environmental

and immunological features of affected individuals. Nevertheless, therapeutic approaches are similar for prevention and treatment of LADA including both specific and nonspecific immunomodulation.

1.8 DOUBLE DIABETES

The attractive term "double diabetes" (DD) applied to the paediatric diabetic population was first introduced by Libman and Becker[56,57] when referring to subjects with an atypical form of diabetes, also called hybrid diabetes, type 1.5 diabetes or LADY (latent autoimmune diabetes in the youth). The presence of autoimmune markers towards beta cells, namely GAD, IA-2 and IAA, typically define cases of DD in patients with T2D[58,59]. It is difficult to define what type of diabetes these subjects suffer from under the current classification, being classified first as affected by T2D because they are obese and insulin resistant but also as affected by T1D because of presence of autoantibodies to beta cells. There is no doubt that these subjects present with an overlapping phenotype of both T2D and T1D. In the adult population these subjects are usually defined as affected by latent autoimmune diabetes in the adult (LADA)[60,61,62]. Such definition is generally based on autoantibody positivity, age at onset (>35 years) and insulin-independence following diagnosis of hyperglycaemia for a period of at least six months.

Several studies have demonstrated a more aggressive course of the disease in LADA subjects characterized by failure of oral hypoglycaemic therapy and progressive beta cell loss leading to insulin dependency usually within five years of diagnosis in subjects with more than one autoantibody to beta cells and when aged 35-45 years[63]. LADA is found in approximately 10% of all cases of T2D, however insulin resistance and obesity are not main features of LADA subjects[64] whereas they are in DD. Therefore it looks like that LADA represents one end of a rainbow of autoimmune

diabetes which is distinguishable from classical T1D only because it is diagnosed in adulthood and presents with some clinical, anthropometric and metabolic features usually associated with T2D.

Despite obesity and metabolic syndrome being on the increase, in particular among Hispanic and Afro-Americans but also in Caucasian youths[65], very little is known about the prevalence of DD. The prevalence and significance of autoimmune markers in children who clinically present with T2D needs to be established in different populations. In particular it needs to be established whether autoantibody-positive youths with DD progress more rapidly to insulin dependence than those with T2D without autoantibodies to beta cells. This is also relevant because these youths may be at increased risk for complications associated with loss of beta cell function, including hypoglycaemia, ketoacidosis, difficult management of the disease and microvascular complications[66], in addition to macrovascular complications typically associated with T2D[67].

CHAPTER 2: PANCREAS

2.1 STRUCTURE OF PANCREAS

The pancreas is a mixed exocrine and endocrine gland. It is an elongated conical organ about 12-15 cm in length, lying laterally to the rear of the upper right hand side of the abdominal cavity. It has been descriptively divided into a head, a body and a tail. The main pancreatic duct runs the length of the gland and empties into the upper duodenum, together with the duct from the liver and gallbladder. The pancreas is encapsulated by loose connective tissue and is divided into lobules by reticular septae. The exocrine and endocrine functions of the gland are carried out by two histologically distinct subunits.

The exocrine portion is organised as a tubuloalveolar gland, acini empty into centroacinar ducts which conduct to progressively larger intralobular ducts, interlobular ducts and eventually to the main pancreatic duct.

The endocrine function is performed by clusters of cells called islets of Langerhans, which are separated from the tubuloalveolar components by a loose reticular capsule. The islets are richly vascularised by a network of fenestrated capillaries. The course of the capillaries allows a degree of autoregulation of the pancreas in that small intralobular arteries first supply the capillary network of the islets, then subsequently invest the nearby acini, allowing autocrine control of pancreatic function. Pancreatic secretion is regulated by intestinal hormones or directly by blood glucose levels. In addition there is a degree of modulation provided by sympathetic inervation of the blood vessels and secretory units.

2.1.1 ENDOCRINE PANCREAS

The part of the pancreas with endocrine function is made up of a million cell clusters called islets of Langerhans. There are four main cell types in the islets. They are relatively difficult to distinguish using standard staining techniques, but they can be classified by their secretion: alpha cells secrete glucagon, beta cells secrete insulin, delta cells secrete somatostatin and gastrin and PP cells secrete pancreatic polypeptide.
The islets are a compact collection of endocrine cells arranged in clusters and cords and are crisscrossed by a dense network of capillaries. The capillaries of the islets are lined by layers of endocrine cells in direct contact with vessels, and most endocrine cells are in direct contact with blood vessels, by either cytoplasmic processes or by direct apposition[68].

A or alpha cells. They make up 15-20% of the cells in the islets. They are responsible for synthesizing and secreting the peptide hormone glucagon, which elevates the glucose levels in the blood. In rodents alpha-cells are located in the periphery of the islets, in humans the islet arcitechture is generally less organized and alpha-cells are frequently observed inside the islets as well. In the electron microscope alpha-cells can be identified by their characteristic granules with a large dense core and a small white halo.

B or beta cells. Beta cells are a type of cell in the pancreas in areas called the islets of Langerhans. They make up 65-80% of the cells in the islets. Beta cells make and release insulin, a hormone that controls the level of glucose in the blood. There is a baseline level of insulin maintained by the

pancreas, but it can respond quickly to spikes in blood glucose by releasing stored insulin while simultaneously producing more. The response time is fairly quick, taking approximately 10 minutes.

Apart from insulin, beta cells release C-peptide, a byproduct of insulin production, into the bloodstream in equimolar quantities. C-peptide helps to prevent neuropathy, and other symptoms of diabetes related to vascular deterioration. Measuring the levels of C-peptide can give a practitioner an idea of the viable beta cell mass.

Beta cells also produce amylin, also known as IAPP, islet amyloid polypeptide. Amylin functions as part of the endocrine pancreas and contributes to glycemic control. Amylin's metabolic function is now somewhat well characterized as an inhibitor of the appearance of nutrient in the plasma.

D or delta cells. Delta cells are somatostatin producing cells. They can be found in the stomach, intestine and the Islets of Langerhans in the pancreas.

In rodents delta cells are located in the periphery of the islets; in humans the islet architecture is generally less organized and delta-cells are frequently observed inside the islets as well. In the electron microscope, delta cells can be identified as cells with smaller and slightly more compact granules than beta cells.

PP cells. PP cells are pancreatic polypeptide producing cells in the islets of Langerhans. They have a polygonal shape and, using an electron microscope, PP cells have been found to be up to 140 nm in diameter.

Epsilon cells. Epsilon cells are endocrine cells found in the Islets of Langerhans and produce the hormone ghrelin. They were recently discovered in mice.

2.1.2 EXOCRINE PANCREAS

Both the acini and the ductal epithelium contribute to pancreatic secretion. Ductal cells are simple squamous or low cuboidal in the intercalated ducts, progressively increasing in height as the diameter of the duct increases, becoming tall columnar in the main pancreatic duct. Occasional goblet cells are interspersed amongst the ductal cells in the larger ducts. The ductal cells are stimulated by the hormone secretin from the gastro intestinal tract to secrete bicarbonate ions and water. This alkaline secretion neutralises the acidic chyme from the stomach, bringing the pH into the optimal range for the action of the pancreatic enzymes within the duodenum.

The pancreatic acini are composed of pyramidal secretory cells, with apical zymogen granules and an extensive basal Golgi complex. The acinar cells secrete an extensive range of digestive enzymes which break down specific components of the chyme as part of the digestive process. Many of the enzymes are secreted as inactive precursors to prevent autodegradation during storage in the zymogen granules and to protect the pancreas after

their release. They are activated in the small intestine by gastrointestinal enteropeptidases[69].

2.2 EMBRYOLOGICAL DEVELOPMENT

The pancreas forms from the embryonic foregut and is therefore of endodermal origin. Pancreatic development begins the formation of a ventral and dorsal anlage (or buds). Each structure communicates with the foregut through a duct.

Differential rotation and fusion of the ventral and dorsal pancreatic buds results in the formation of the definitive pancreas. As the duodenum rotates to the right, it carries with it the ventral pancreatic bud and common bile duct. Upon reaching its final destination, the ventral pancreatic bud fuses with the much larger dorsal pancreatic bud. At this point of fusion, the main ducts of the ventral and dorsal pancreatic buds fuse, forming the duct of Wirsung, the main pancreatic duct (see figure 3).

Differentiation of cells of the pancreas proceeds through two different pathways, corresponding to the dual endocrine and exocrine functions of the pancreas. In progenitor cells of the exocrine pancreas, important molecules that induce differentiation include follistatin, fibroblast growth factors, and activation of the Notch receptor system. Development of the exocrine acini progresses through three successive stages. These include the predifferentiated, protodifferentiated, and differentiated stages, which correspond to undetectable, low, and high levels of digestive enzyme activity, respectively.

Progenitor cells of the endocrine pancreas arise from cells of the protodifferentiated stage of the exocrine pancreas. Under the influence of neurogenin-3 and Isl-1, but in the absence of Notch receptor signaling, these cells differentiate to form two lines of committed endocrine precursor cells. The first line, under the direction of Pax-6, forms alfa and gamma cells, which produce the peptides glucagon and pancreatic polypeptide, respectively. The second line, influenced by Pax-4, produces beta and delta cells, which secrete insulin and somatostatin, respectively. Insulin and glucagon can be detected in the fetal circulation by the fourth of fifth month of fetal development[70].

2.2.1 ENDOCRINE PANCREAS DEVELOPMENT

The presumptive islet cell clumps migrate away from the tubules into the stroma of the developing gland, while new clumps continue to form and bud off. The islets expand through proliferation of the islet cell precursors and by merging of cell clumps in close proximity. During week 10 type A cells differentiate. At the same time, angiogenesis begins within the mesenchyme and the primitive mesenchymal cells become more fibroblast like. During week 11 type D cells appear within the islets, which by now are migrating towards the capillaries forming within the stroma. During week 13 the type B cells appear. The accumulation of each cell type within the islet is not a stepwise event and there are initially very few cells of each phenotype. The rate of appearance of each cell type and their final numbers appears to be under mesenchymal control. By week 13 a fine capillary network has formed within the more developed islets and by

week 17 the B cells begin to secrete insulin, which they continue to do until birth. There is a transient secretion of the A cells between weeks 20 to 24. Differentiated cells with an endocrine phenotype are also detected within the epithelium of the ducts and acini from week 17 onwards.

2.2.2 EXOCRINE PANCREAS DEVELOPMENT

By week 12, the interlobular ducts are established, forming the pattern of the future lobular structure of the pancreas. More distally are the intralobular ducts leading to an extensive network of intercallated ducts with developing acini at their terminal ends. There is a developing vascular system surrounding the primitive tubules and the stroma consists of a uniform cellular mesenchyme.

Differentiation of the primitive acini begins around week 12. Pyramidal cells arise within the terminal cell clumps and become grouped around presumptive centroacinar cells as the acini dilate. The epithelial cells of the exocrine system express glycogen as they differentiate. Glycogen concentrations are higher in the acinar cells, progressively reducing towards the epithelial cells of the larger ducts. The stroma gains a large amount of connective tissue and fibroblasts differentiate from the mesenchymal cells. Between weeks 14 and 20, the developing acini expand and the stroma is reduced, so that the lobular appearance of the gland becomes better defined. As the acini mature, the intracellular glycogen is reduced and there is a corresponding increase in the number of zymogen granules. By week 16, the first mature acini are formed. Glycogen is absent

from the ducts and from most of the acinar cells by 21 weeks, the number and size of the acinar zymogen granules continues to increase until birth.

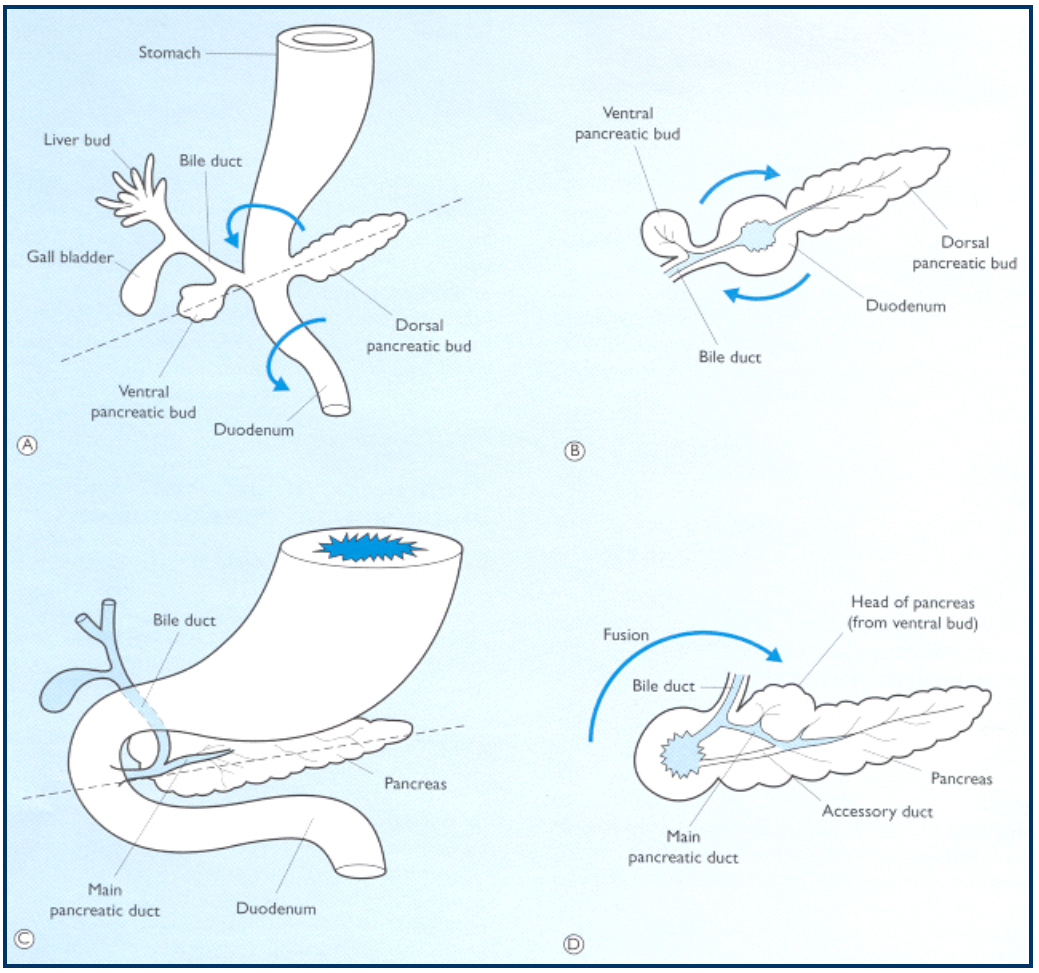


Figure 3. Schematic drawings of the development of the human pancreas at 6 weeks Ⓐ and Ⓑ and 8 weeks Ⓒ and gestation Ⓓ.
Growth and rotation of the duodenum (indicated by arrows in Ⓐ and Ⓑ) cause movement of the ventral pancreatic bud towards the dorsal bud and their eventual fusion Ⓓ. Union of the distal part of the dorsal pancreatic duct and the entire ventral pancreatic duct forms the main pancreatic duct. The proximal part of the dorsal pancreatic duct usually disappears but it may persist as an accessory duct Ⓓ.

2.3 INSULIN PRODUCTION & SECRETION

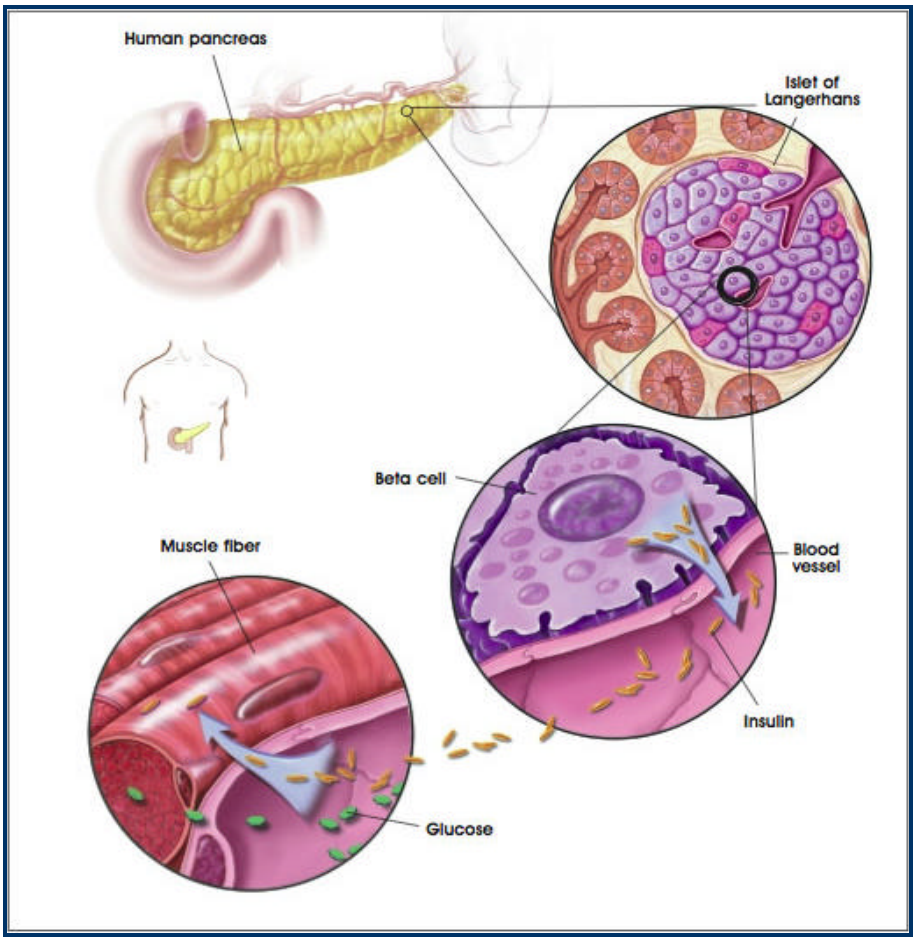


Figure 4. Insulin Production in the Human Pancreas.

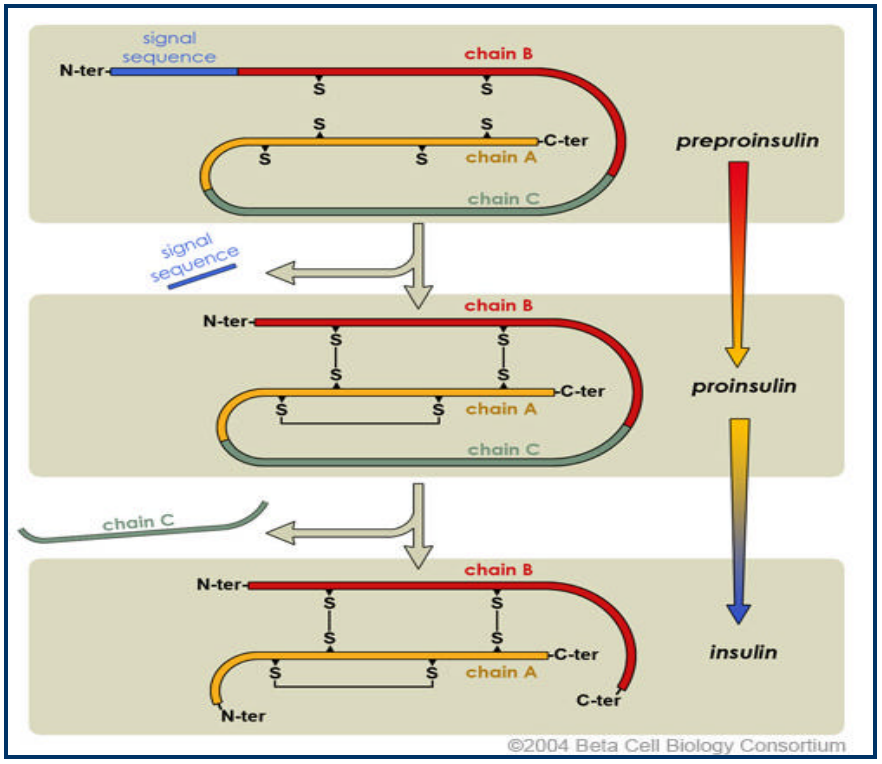
The pancreas is located in the abdomen, adjacent to the duodenum (the first portion of the small intestine). A cross-section of the pancreas shows the islet of Langerhans which is the functional unit of the endocrine pancreas. Encircled is the beta cell that synthesizes and secretes insulin. Beta cells are located adjacent to blood vessels and can easily respond to changes in blood glucose concentration by adjusting insulin production. Insulin facilitates uptake of glucose, the main fuel source, into cells of tissues such as muscle.

Insulin is a hormone that is exclusively produced by pancreatic beta cells (see figure 4). Insulin is a small protein and is produced as part of a larger protein to ensure it folds properly. In the protein assembly of insulin, the messenger RNA transcript is translated into an inactive protein called *preproinsulin.* Preproinsulin contains an amino-terminal signal sequence that is required in order for the precursor hormone to pass through the membrane of the endoplasmic reticulum (ER) for post-translational processing. The post-translational processing clips away those portions not needed for the bioactive hormone. Upon entering the ER, the preproinsulin signal sequence is proteolytically removed to form *proinsulin*. Once the post-translational formation of three vital disulfide bonds occurs, specific peptidases cleave proinsulin. The final product of the biosynthesis is mature and active insulin (see figure 5). Finally, insulin is packaged and stored in secretory granules, which accumulate in the cytoplasm, until release is triggered.

Type 2 glucose transporters (GLUT2) mediate the entry of glucose into beta cells[71] (see figure 6). As the raw fuel for glycolysis, the universal energy-producing pathway, glucose is phosphorylated by the rate-limiting enzyme glucokinase. This modified glucose becomes effectively trapped within the beta cells and is further metabolized to create ATP. The increased ATP:ADP ratio causes the ATP-gated potassium channels in the cellular membrane to close up, preventing potassium ions from being shunted across the cell membrane. The ensuing rise in positive charge inside the cell leads to depolarization of the cell. The net effect is the activation of voltage-gated calcium channels, which transport calcium ions

into the cell. The brisk increase in intracellular calcium concentrations triggers export of the insulin-storing granules by a process known as exocytosis. The ultimate result is the export of insulin from beta cells and its diffusion into nearby blood vessels. Insulin release is a biphasic process. The initial amount of insulin released upon glucose absorption is dependent on the amounts available in storage. Once depleted, a second phase of insulin release is initiated. This latter release is prolonged since insulin has to be synthesized, processed, and secreted for the duration of the increase of blood glucose. Furthermore, beta cells also have to regenerate the stores of insulin initially depleted in the fast response phase[72].

Insulin molecules circulate throughout the blood stream until they bind to their associated (insulin) receptors. The insulin receptors promote the uptake of glucose into various tissues that contain type 4 glucose transporters (GLUT4)[73] (see figure 7). Such tissues include skeletal muscles (which burn glucose for energy) and fat tissues (which convert glucose to triglycerides for storage). The initial binding of insulin to its receptor initiates a signal transduction cascade that communicates the message delivered by insulin: remove glucose from blood plasma. Among the wide array of cellular responses resulting from insulin activation, the key step in glucose metabolism is the immediate activation and increased levels of GLUT4 glucose transporters. By the facilitative transport of glucose into the cells, the glucose transporters effectively remove glucose from the blood stream. Insulin binding results in changes in the activities



and concentrations of intracellular enzymes such as GLUT4. These changes can last from minutes to hours.

Figure 5. Insulin Synthesis.

Insulin production involved intermediate steps. Initially, preproinsulin is the inactive that is secreted into the endoplasmic reticulum. Post-translational processing clips the N-terminal signal sequence and forms the disulfide bridges. Lastly, the polypeptide is clipped at two positions to release the intervening chain C. This and active insulin are finally packaged into secretory granules for storage.

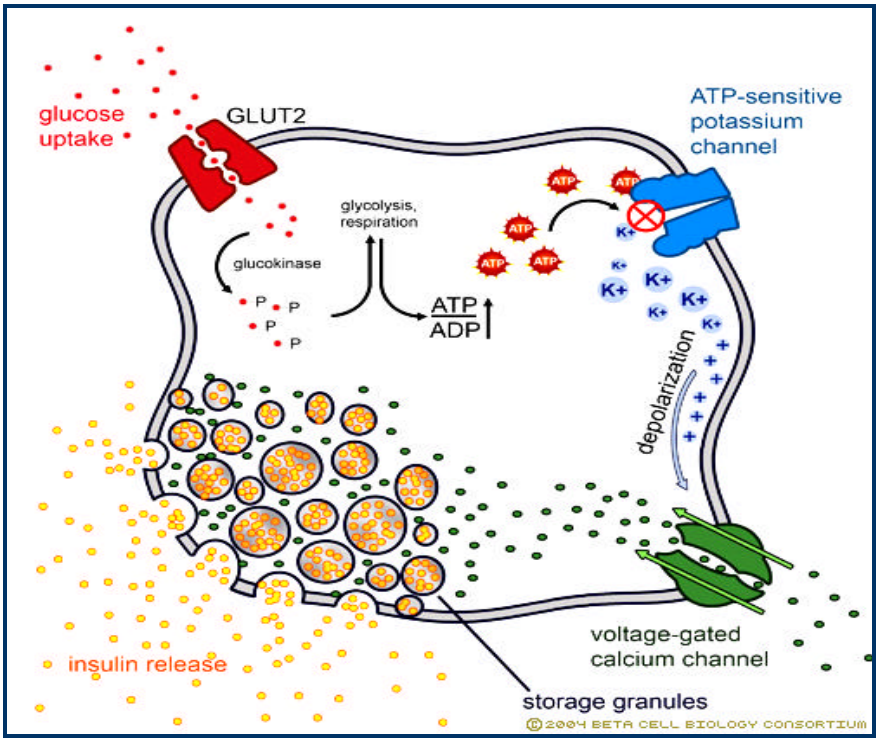


Figure 6. Insulin secretion.

Insulin secretion in beta cells is triggered by rising blood glucose levels. Starting with the uptake of glucose by the GLUT2 transporter, the glycolytic phosphorylation of glucose causes a rise in the ATP:ADP ratio. This rise inactivates the potassium channel that depolarizes the membrane, causing the calcium channel to open up allowing calcium ions to flow inward. The ensuing rise in levels of calcium leads to the exocytotic release of insulin from their storage granule.

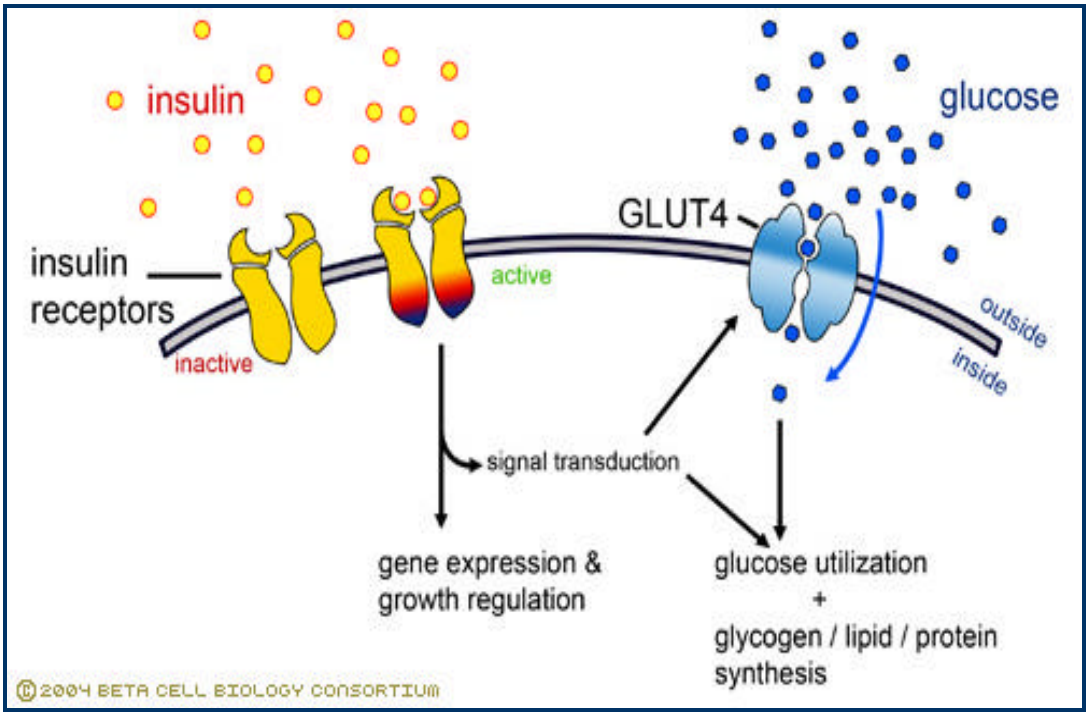


Figure 7. Insulin-mediated glucose uptake

Insulin binding to the insulin receptor induces a signal transduction cascade which allows the glucose transporter (GLUT4) to transport glucose into the cell.

2.4 DISEASES OF THE PANCREAS

Because the pancreas is a storage depot for digestive enzymes, injury to the pancreas is potentially very dangerous. Diseases associated with the pancreas, togheter with diabetes mellitus, include:

a) Pancreatitis. Pancreatitis is inflammation of the pancreas. There are two forms of pancreatitis, which are different in causes and symptoms, and require different treatment. Acute pancreatitis is a rapidly-onset inflammation of the pancreas, most frequently caused by alcoholism or gallstones. Chronic pancreatitis is a long-standing inflammation of the pancreas.

b) Exocrine pancreatic insufficiency. Exocrine pancreatic insufficiency (EPI) is the inability to properly digest food due to a lack of digestive enzymes made by the pancreas. EPI is found in humans afflicted with cystic fibrosis and Shwachman-Diamond Syndrome. It is caused by a progressive loss of the pancreatic cells that make digestive enzymes. Chronic pancreatitis is the most common cause of EPI in humans. Loss of digestive enzymes leads to maldigestion and malabsorption of nutrients.

c) Cystic fibrosis. Cystic fibrosis, also known as mucoviscidosis, is a hereditary disease that affects the entire body, causing progressive disability and early death. There is no cure for cystic fibrosis, and most affected individuals die young from lung failure. Cystic fibrosis is caused by a mutation in the cystic fibrosis transmembrane conductance regulator

(CFTR) gene. The product of this gene helps create sweat, digestive juices, and mucus. Although most people without CF have two working copies of the CFTR gene, only one is needed to prevent cystic fibrosis. Cystic fibrosis develops when neither gene works normally. Therefore, it is considered an autosomal recessive disease. The name cystic fibrosis refers to the characteristic 'fibrosis' (tissue scarring) and cyst formation within the pancreas. Cystic fibrosis causes irreversible damage to the pancreas, which often results in painful inflammation.

d) Pseudocysts. A pancreatic pseudocyst is a circumscribed collection of fluid rich in amylase and other pancreatic enzymes, blood and necrotic tissue, typically located in the lesser sac.

e) Congenital malformations.

- Pancreas divisum

Pancreas divisum is a malformation in which the pancreas fails to fuse together. It is a rare condition that affects only 6% of the world's population and of these few only 1% ever have symptoms that require surgery.

- Annular pancreas

Annular pancreas is characterized by a pancreas that encircles the duodenum. It results from an embryological malformation in which the early pancreatic buds undergo inappropriate rotation and fusion, which can lead to small bowel obstruction.

f) Pancreatic cancer. Pancreatic cancer is a malignant tumor of the pancreas. Each year about 33,000 individuals in the United States are diagnosed with this condition, and more than 60,000 in Europe. Depending on the extent of the tumor at the time of diagnosis, the prognosis is generally regarded as poor, with few victims still alive five years after diagnosis, and complete remission still extremely rare. About 95 % of pancreatic tumors are adenocarcinomas. The remaining 5 % include other tumors of the exocrine pancreas (e.g., serous cystadenomas), acinar cell cancers, and pancreatic neuroendocrine tumors such as insulinomas. These tumors have a completely different diagnostic and therapeutic profile, and generally a more favorable prognosis.

g) Zollinger-Ellison syndrome. Zollinger-Ellison syndrome is a collection of findings in individuals with gastrinoma, a tumor of the gastrin-producing cells of the pancreas. Unbridled gastrin secretion results in elevated levels of the hormone, and increased hydrochloric acid secretion from parietal cells of the stomach. It can lead to ulceration and scarring of the stomach and intestinal mucosa.

CHAPTER 3: THE NON-OBESE DIABETIC MOUSE (NOD MOUSE)

3.1 ANIMAL MODELS FOR HUMAN TYPE 1 DIABETES

Much of our present knowledge regarding the pathogenesis, treatment and prevention of T1D would never have been acquired without the study of animal models of diabetes.

The main in vivo models of T1D may be divided in two groups: 1) induced diabetes models i.e. through pancreasectomy or through treatment with chemicals such as Alloxan or Streptozotocin or through viral infections and 2) spontaneous diabetes models i.e. the Bio Breeding (BB) rat and the Non Obese Diabetic (NOD) mouse.

3.1.1 INDUCED ANIMAL MODELS OF TYPE 1 DIABETES

Many animal species have been used and some continue to be used as inducible models of diabetes. These include the Chinese hamster[74], mini pigs[75], dogs[76] and even non human primates[77]. The fact that diabetes is artificially induced in these animal models causes problems as these models give no indication to the disease process. If we are going to attempt intervention in man, understanding the disease process is very important. In the studies used for the discovery of insulin, dogs were extensively used, their size made them very useful animals on which to carry out surgical procedures such as pancreasectomy, a procedure that was frequently used in early T1D research.

However, today their size make them less useful, because of the expensive facilities required to keep them. A further point, which certainly needs to be considered, is te very low acceptability by the public for use of many species of animals to be used as disease models. This is expecially true regarding cats, dogs and primates. Much easier is to work with rodents. Rats and mice are generappy preferred as they are relatively docile, easy to handle, non expensive to maintain, have a short gestational period and life span allowing the study of many generations in a relatively short period of time.

There are also several chemically induced rodent models of diabetes using chemials known to be toxic to beta cells and/or capable to produce diabetes. These include the commonly used Streptozotocin and Alloxan induced diabetic mouse models of diabetes. These chemicals are known to be rapidly diabetogenic infact the toxicity of Streptozotocin to pancreatic beta cells occurs within 15 minutes from its administration[78]. Because the onset of diabetes in these treated mice is extremely rapid they obviously cannot be regarded as models of spontaneous disease nor do they display all of the pathological signs of prediabetes, such as autoantibody formation or gradual insulitis[79,80].

3.1.2 SPONTANEOUS ANIMAL MODELS OF TYPE 1 DIABETES

Two main spontaneous models of T1D are widely used, the BB rat and the NOD mouse. The BB rat was established at the Bio-Breeding Laboratories of Canada Ltd in 1974 and shares many of the characteristics of T1D found in humans. However in this animal model has been observed lymphopenia

which is obviously a severe problem in a model of an autoimmune disease[81]. BB rats are also very difficult to keep, requiring special housing and handling procedures, as they are prone to infections, particulary respiratory tract infections, which are usually fatal. Another rodent model., which was initially discovered in 1974 but not widely available until much later, is the NOD mouse[82]. This animal is currently regarded as the mest available spontaneous model for studying the pathogenesis of human T1D as this strain develops a form of diabetes very similar to that foud in humans.

3.2 THE NOD MOUSE

The NOD mouse is the best available spontaneous animal model of human T1DM since it develops an autoimmune form of the disease comparable to the disease that occurs in humans[83]. Testing therapeutic agents to find out whether diabetes could be delayed or prevented in these mice may provide valuable results for disease prevention in humans. Indeed, the Immunology of Diabetes Society in their guidelines indicate that trials in humans to prevent the disease should be carried out after successful attempts are obtained with the proposed approach in NOD mice.

The NOD mouse spontaneously develops T cell-mediated autoimmune diabetes leading to the destruction of insulin secreting beta cells[84].

A pre-diabetic state is characterised by the infiltration of macrophages and lymphocytes into the pancreatic islets (insulitis). This step precedes the complete destruction of the pancreatic beta cell mass.

Insulitis begins at approximately 4-6 weeks of age and diabetes develops between 12-18 weeks predominantly in females.

This animal model develops glutamic acid decarboxylase antibodies (GAD)[85] islet cell antibodies (ICA) and insulin autoantibodies (IAA) [86] which have been also involved in T1D development in humans.

The NOD mouse strain was originally established at the Shionogi Laboratories in Japan from an inbred cataract Shionogi (Cts) strain. The work was carried out with the aim to develop a sub-strain with raised blood glucose levels in order to study the effect of a 'diabetes-like' condition on cataract development. In 1974 one of the Cts female mice was recognised as not only having raised blood glucose levels but as actually displaying the clinical picture of T1D. Unfortunately, however, this mouse died before producing offspring. The sub-line was maintained and monitored for a further six years before another mouse with the same features was found. This mouse was successfully bred and resulted in the establishment of the NOD mouse in 1980 [87].

3.3 FEATURES OF NOD MOUSE

In the NOD mouse the course of the disease follows a pattern similar to that found in human T1D. A pre-diabetic state is characterised by the infiltration of macrophages and lymphocytes into the pancreatic islets (insulitis). Clinical signs of the disease, which are common to both NOD mice and humans include polydipsia, polyuria, glycosuria, polyphagia, weight loss, lassitude, coma, and death if left untreated[88,89]. Insulitis is

observed in NOD mice from the fourth week of age with a progressive destruction of the beta cells. Disease onset usually is localized around the tenth week of age. The NOD mouse also shows histological signs of thyroiditis, sialitis, parathyroiditis and adrenalitis[90].

Mice are monitored weekly from 10 weeks of age for urinary glucose using Diabur-test 500 strips (Roche) to determine the status of the diabetes. In a mouse that has no diabetes, no glucose in the urine is detectable. At diabetes onset, (which to date has not been noted any earlier than 10 weeks of age in our own colony) a glucose concentration as high as 5% (280 mmol/l) is detectable; however, any value over 1% (56mmol/l) for 2 weekly readings confirms the diagnosis of diabetes.

In our experience we have noted that an animal that displays glycosuria levels of 56mmol/l or greater consistently for 2 consecutive weeks never reverts to a non-diabetic state. Animals that are diabetic are normally culled after 2 weeks, although at this stage the disease does not normally cause them undue distress. Our group has been instrumental in describing the optimal husbandry and experimental settings for the use of these mice. We have offered practical advice to prospective colony keepers , written guidelines on the standardisation of experiments in the mice[91] and developed methods to compare data from colonies around the world[92].

Some NOD mice develop both diabetes and cataracts and so have a dual function in that they can serve as diabetic mice with ocular complications. Attempts to prevent cataract formation can therefore be made as can investigations into its cause in these mice. Cataract formation is a common

complication in long-term diabetes in humans and although these mice develop cataracts before diabetes, they normally have slightly elevated blood glucose levels even if they do not develop full-blown diabetes. It may be that this increase in blood glucose is sufficient to cause cataracts or it may be due to a biochemical defect in these mice or a combination of both. It is therefore considered important to retain this genetic defect in order for it to be investigated further. In our unit we reported a substrain of NOD mice which developed spontaneous cataract[93]. A sub-line of animals of this type may be useful for studies aimed at modifying the course of this eye complication, which has a huge impact on the patient's quality of life.

3.4 THE NOD MOUSE COLONY AT QUEEN MARY UNIVERSITY OF LONDON

Our NOD/Ba colony was established in 1987 at the Animal House at Charterhouse, St Bartholomew's Medical School and its was originally derived from Dr. E.Leiter's laboratory (Bar HArbor, ME, USA).

The colony is housed in a purpose built area and maintained strictly according the international (NIH Principles of laboratory animal care, 1985) and UK (HMSO, Animal Scientific Procedures, 1986) guidelines for animal care. There is a stable cumulative incidence of diabetes approximately 60% in female and 15% in male mice at 30 weeks of age (see figure 8).

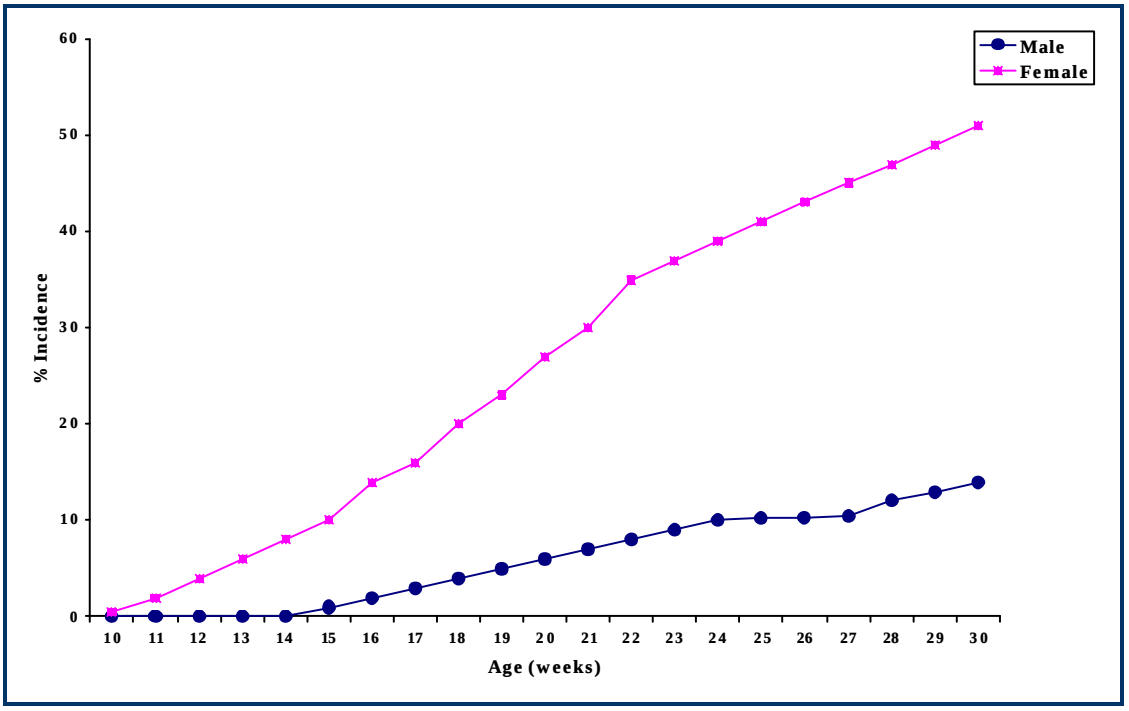


Figure 8 . Graphical illustration of the cumulative incidence of T1D in NOD/Ba mice from 10 to 30 weeks of age.
The cumulative incidence of diabetes in male(n=256) and female (n=264) NOD/Ba stock mice up to 32 weeks of age from the NOD/Ba mouse colony at Queen Mary College, London.

Also, the animals were born and grown in individually ventilated cage rack with exhaust system (Sealsafe IVC) and on the relevant safety standards. The Sealsafe IVC is designed to function in an environment that is free of atmospheric agents and in a controlled temperature. Temperature is maintained at 21 degree, relative humidity at 50% and there are 15 air changes per hour and 14 hours of light and 10 hours of darkness per day. Like all NOD mice, NOD/Ba mice are albino (see figure 9).

Figure 9. A photographic illustration of a NOD mouse

Typical birth weight is of around 1.8 grams and with adult body weight of around 25-27 grams for females and 35 grams for males (see figure 10). Pubery is at around 42 day of age and gestation time is aroud 20-22 days. All the experiments were carried out under the terms of the Animal Scientific Procedures Act of 1986 under UK Government (Home Office-project licence PPL70-5962).

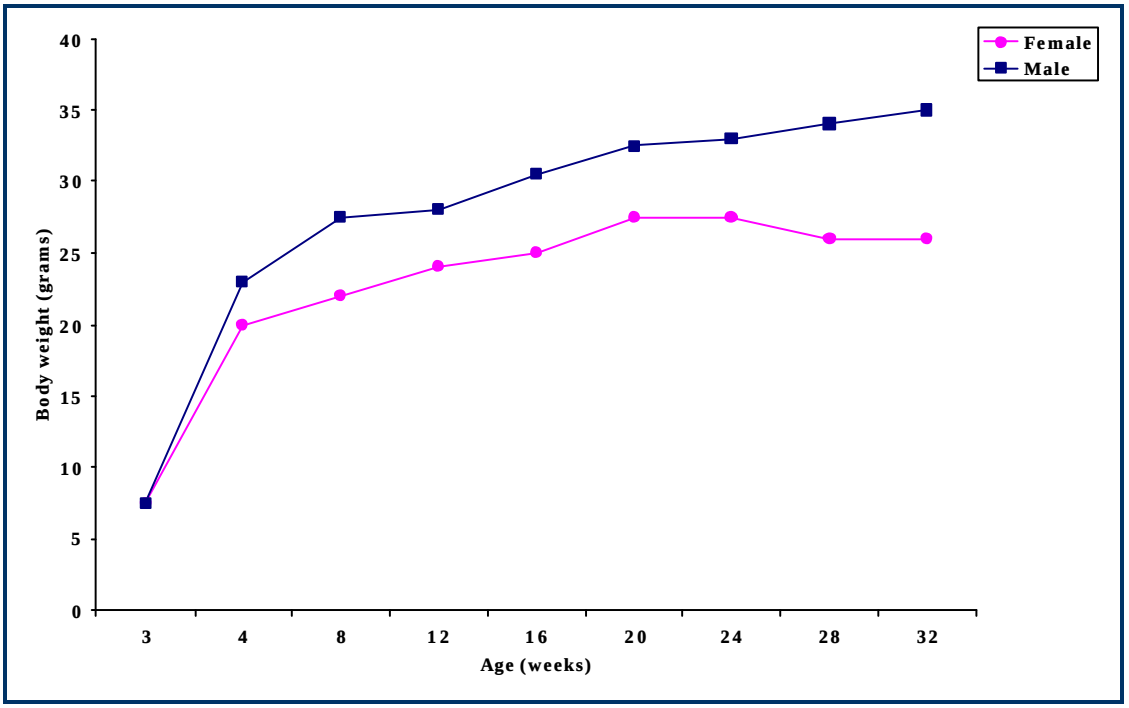


Figure 10. Graphical illustration of the body weight of male and female NOD/Ba stock mice from 3 to 32 weeks of age. Body weight of female and male NOD/Ba stock mice from 3 to 32 weeks of age from colony at Queen Mary College, London. Male NOD/Ba mice weight on average approximately 5-10 grams more than female NOD/Ba mice at the majority of ages.

CHAPTER 4: ISOLATION OF MESENCHYMAL CELLS FROM BONE MARROW OF NOD MICE

4.1 BACKGROUND

Research on stem cells is a promising area of science leading to investigate the possibility of cell-based therapies to treat disease, often referred to as regenerative or reparative medicine.

Stem cells are one of the most fascinating areas of biology today but like many expanding fields of scientific inquiry, research on stem cells raises scientific questions as rapidly as it generates new discoveries.

Stem cells have the remarkable potential to develop into many different cell types in the body. Serving as a sort of repair system for the body, they can theoretically divide without limit to replenish other cells.

Stem cells are exciting to physicians, scientists and patients because of their potential to develop into many different cell types and tissues that can possibly be used to treat large numbers of patients with a variety of conditions including cardiovascular disease, diabetes, neurodegenerative disorders and spinal cord injuries. To scientists, stem cells appear to offer "unprecedented opportunities for developing new medical therapies for debilitating diseases and a new way to explore fundamental questions of biology"[94].

Until quite recently it was thought that stem cells found in adult tissues and organs could differentiate only into the particular type of cells that make up the organ where the stem cell resides. "Adult" in this context simply means cells from tissues or organs that already exist as such, and in

which each cell has a full, diploid, complement of genetic material (e.g. cells from the skin, liver, kidneys and bone marrow). It was widely believed that neuronal stem cells could only make neurons, hematopoietic stem cells could only make blood cells and so on. In other words, it was thought that the cells lacked plasticity and were inherently unable to transdifferentiate into other cell types. However, over the past few years it has been repeatedly demonstrated that stem cells originating from one organ or tissue can develop into cell types of another tissue[95,96]. This has been shown in both animals and humans[97,98].

The last decade has seen an exponential increase in the number of manuscripts on other stem cell types, including skin, liver, pancreas, brain, lung, intestine, skeletal muscle, cardiac, cord blood, etc. and over the last year, dozens of published articles have added to our knowledge of the substantial abilities of adult stem cells. Several new reports highlight the pluripotent ability of adult stem cells from new sources including a new isolate from bone marrow[99] and umbilical cord blood[100]. These references also indicate the ability for extensive proliferation of adult stem cells, especially those derived from cord blood.

T1D is caused by genetic and environmental factors. More than 17 different chromosomal regions have been implicated in contributing to susceptibility to the disease in the NOD mouse, an animal model that shares many characteristics with the human disease.

Replacement of the insulin-producing beta cells represents the ultimate treatment for T1D. Recent advances in islet transplantation underscore the urgent need for developing alternatives to human tissue donors, which are

scarce. Two possible approaches are the expansion of differentiated beta cells by reversible immortalization and the generation of insulin-producing cells from embryonic or adult stem cells. It is possible that new insights into endocrine pancreas development will ultimately lead to manipulation of progenitor-cell fate towards the beta cell phenotype of insulin production, storage and regulated secretion. Both allogeneic and autologous surrogate beta cells are likely to require protection from recurring autoimmunity. This protection might take the form of tolerization, cell encapsulation, or cell engineering with immunoprotective genes. If successful, these approaches could lead to widespread cell replacement therapy for T1D[101, 102].

The pancreas and potential to treat diabetes has been highlighted in several recent studies[103]. Several published references in the past years addressed the possible existence of a pancreatic stem cell. One reference indicates that regeneration of beta cells in the pancreas is solely due to existing beta cells[104] while another reference indicates the existence of a multipotent progenitor within pancreas that can form either pancreatic or neural cell lineages[105]. Another group has provided evidence of transdifferentiation of bone marrow-derived stem cells into pancreatic cells[106]. A Harvard group has shown that pancreatic islet progenitors can engraft in mice[107] and has also shown permanent reversal of diabetes in mice[108].

4.2 DEFINITION OF STEM CELLS

Stem cellular structures are cells found in most multi-cellular organisms. They are capable of retaining the ability to reinvigorate themselves through mitotic cell division and can differentiate into a diverse range of specialized cell types. Research in the stem cell field grew out of findings by Canadian scientists Ernest A. McCulloch and James E. Till in the 1960 [109,110].

The two broad types of mammalian stem cells are: *embryonic stem cells* that are found in blastocysts, and *adult stem cells* that are found in adult tissues. In a developing embryo, stem cells can differentiate into all of the specialized embryonic tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing specialized cells, but also maintain the normal turnover of regenerative organs, such as blood, skin or intestinal tissues.

As stem cells can be grown and transformed into specialized cells with characteristics consistent with cells of various tissues such as muscles or nerves through cell culture, their use in medical therapies has been proposed. In particular, embryonic cell lines, autologous embryonic stem cells generated through therapeutic cloning, and highly plastic adult stem cells from the umbilical cord blood or bone marrow are touted as promising candidates[111].

4.3 CHARACTERISTICS OF STEM CELLS

Stem cells differ from other kinds of cells in the body. All stem cells, regardless of their source, have three general properties:

1) ***They are capable of dividing and renewing themselves for long periods.*** Unlike muscle cells, blood cells, or nerve cells — which do not normally replicate themselves — stem cells may replicate many times. When cells replicate themselves many times over it is called clonogenicity. A starting population of stem cells that proliferates for many months in the laboratory can yield millions of cells. If the resulting cells continue to be unspecialized, like the parent stem cells, the cells are said to be capable of long-term self-renewal.

The specific factors and conditions that allow stem cells to remain unspecialised are of great interest to scientists. It has taken scientists many years of trial and error to learn to grow stem cells in the laboratory without them spontaneously differentiating into specific cell types. For example, it took 20 years to learn how to grow human embryonic stem cells in the laboratory following the development of conditions for growing mouse stem cells. Therefore, an important area of research is understanding the signals in a mature organism that cause a stem cell population to proliferate and remain unspecialised until the cells are needed to repair a specific tissue. Such information is critical for scientists to be able to grow large numbers of unspecialised stem cells in the laboratory for further experimentation.

2) ***They are unspecialised***. One of the fundamental properties of a stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. A stem cell cannot work with its neighbours to pump blood through the body (like a heart muscle cell); it

cannot carry molecules of oxygen through the bloodstream (like a red blood cell); and it cannot fire electrochemical signals to other cells that allow the body to move or speak (like a nerve cell). However, unspecialised stem cells can give rise to specialized cells, including heart muscle cells, blood cells, or nerve cells.

3) ***They can give rise to specialised cell types.*** When unspecialised stem cells give rise to specialized cells, the process is called differentiation. Scientists are just beginning to understand the signals inside and outside cells that trigger stem cell differentiation. The internal signals are controlled by a cell's genes, which are interspersed across long strands of DNA, and carry coded instructions for all the structures and functions of a cell. The external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighbouring cells, and certain molecules in the microenvironment.

There are four types of stem cells:

Totipotent stem cells are produced from the fusion of an egg and sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent. These cells can differentiate into embryonic and extraembryonic cell types.

Pluripotent stem cells are the descendants of totipotent cells and can differentiate into cells derived from any of the three germ layers.

Multipotent stem cells can produce only cells of a closely related family of cells (e.g. hematopoietic stem cells differentiate into red blood cells, white blood cells, platelets, etc.).

Unipotent cells can produce only one cell type, but have the property of self-renewal which distinguishes them from non-stem cells (e.g. muscle stem cells).

4.4 SOURCES OF STEM CELLS

4.4.1 EMBRYONIC STEM CELLS

Embryonic stem cells (ES cells) are stem cells derived from the inner cell mass of an early stage embryo known as a blastocyst (see figure 11). Human embryos reach the blastocyst stage 4-5 days post fertilization, at which time they consist of 50-150 cells. ES cells are pluripotent. This means they are able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. These include each of the more than 220 cell types in the adult body. Pluripotency distinguishes ES cells from multipotent progenitor cells found in the adult; these only form a limited number of cell types. When given no stimuli for differentiation, (i.e. when grown in vitro), ES cells maintain pluripotency through multiple cell divisions. The presence of pluripotent adult stem cells remains a subject of scientific debate, however, research has demonstrated that pluripotent stem cells can be directly generated from adult fibroblast cultures[112].

Because of their plasticity and potentially unlimited capacity for self-renewal, ES cell therapies have been proposed for regenerative medicine and tissue replacement after injury or disease. However, to date, no approved medical treatments have been derived from embryonic stem cell research.

ES cell lines are cultures of cells derived from the epiblast tissue of the inner cell mass (ICM) of a blastocyst or earlier morula stage embryos. A blastocyst is an early stage embryo, approximately four to five days old in humans and consisting of 50–150 cells. ES cells are pluripotent and give rise during development to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. In other words, they can develop into each of the more than 200 cell types of the adult body when given sufficient and necessary stimulation for a specific cell type. They do not contribute to the extra-embryonic membranes or the placenta.

Nearly all research to date has taken place using mouse embryonic stem cells (mES) or human embryonic stem cells (hES). Both have the essential stem cell characteristics, yet they require very different environments in order to maintain an undifferentiated state. Mouse ES cells are grown on a layer of gelatin and require the presence of Leukemia Inhibitory Factor (LIF). Human ES cells are grown on a feeder layer of mouse embryonic fibroblasts (MEFs) and require the presence of basic Fibroblast Growth Factor (bFGF or FGF-2). Without optimal culture conditions or genetic manipulation[113], embryonic stem cells will rapidly differentiate. A human embryonic stem cell is also defined by the presence of several transcription

factors and cell surface proteins. The transcription factors Oct-4, Nanog, and SOX2 form the core regulatory network that ensures the suppression of genes that lead to differentiation and the maintenance of pluripotency[114]. The cell surface antigens most commonly used to identify hES cells are the glycolipids SSEA3 and SSEA4 and the keratan sulfate antigens Tra-1-60 and Tra-1-81. The molecular definition of a stem cell includes many more proteins and continues to be a topic of research[115].

After twenty years of research, there are no approved treatments or human trials using embryonic stem cells. ES cells, being pluripotent cells, require specific signals for correct differentiation. If injected directly into the body, ES cells will differentiate into many different types of cells, causing a teratoma. Differentiating ES cells into usable cells while avoiding transplant rejection are just a few of the hurdles that embryonic stem cell researchers still face[116].

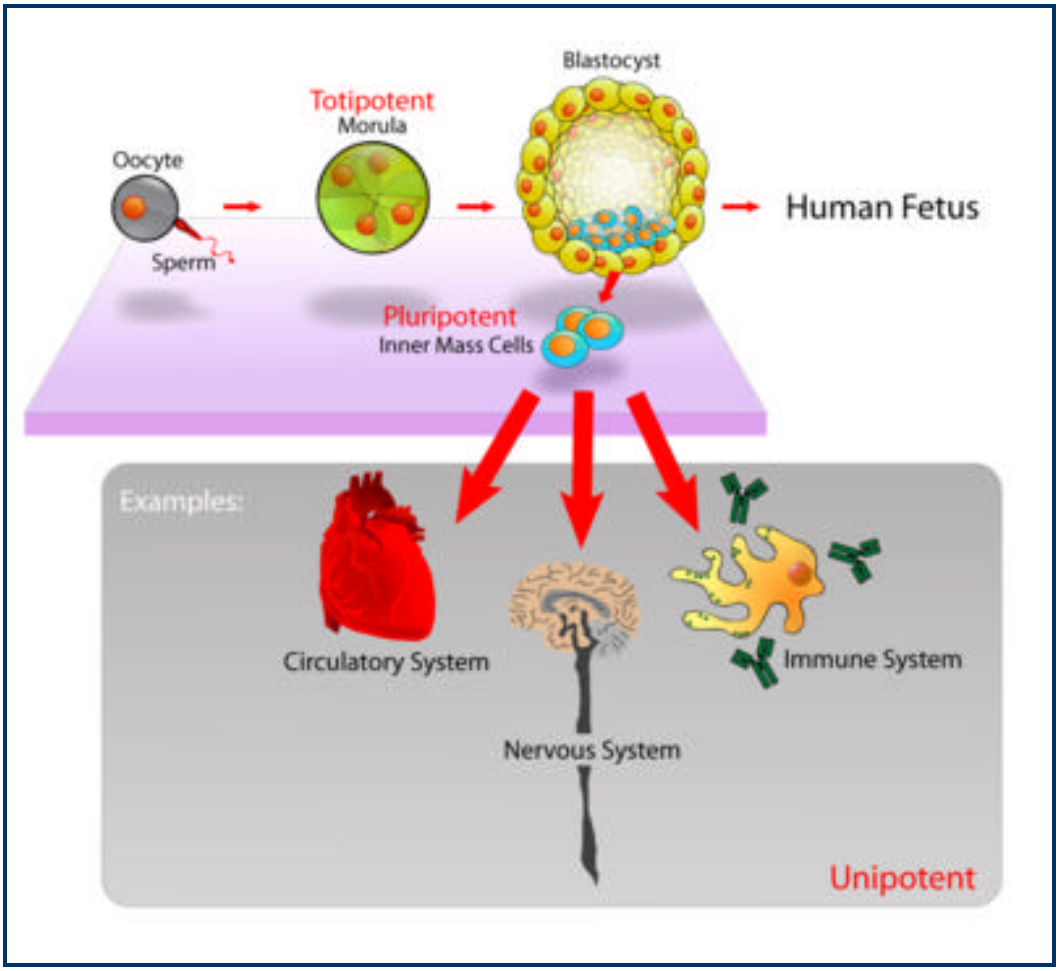


Figure 11. Embryonic stem cells. Pluripotent, embryonic stem cells originate as inner mass cells within a blastocyst. The stem cells can become any tissue in the body, excluding a placenta. Only the morula's cells are totipotent, able to become all tissues and a placenta.

4.4.2 ADULT STEM CELLS

The term adult stem cell refers to any cell which is found in a developed organism that has two properties: the ability to divide and create another cell like itself and also divide and create a cell more differentiated than itself. Also known as somatic (from Greek S? µat??ó?, "of the body") stem cells and germline (giving rise to gametes) stem cells, they can be found in children, as well as adults[117]. Pluripotent adult stem cells are rare and generally small in number but can be found in a number of tissues including umbilical cord blood[118]. Most adult stem cells are lineage-restricted (multipotent) and are generally referred to by their tissue origin (mesenchymal stem cell, adipose-derived stem cell, endothelial stem cell, etc.)[119,120]. Stem cells can be found in all adult and young adult beings. Adult stem cells are undifferentiated cells that reproduce daily to provide certain specialized cells, for example 200 billion red blood cells are created each day in the body from hemopoietic stem cells. Until recently it was thought that each of these cells could produce just one particular type of cell. However in the past few years, evidence has been gathered of stem cells that can transform into several different forms (see figure 12).

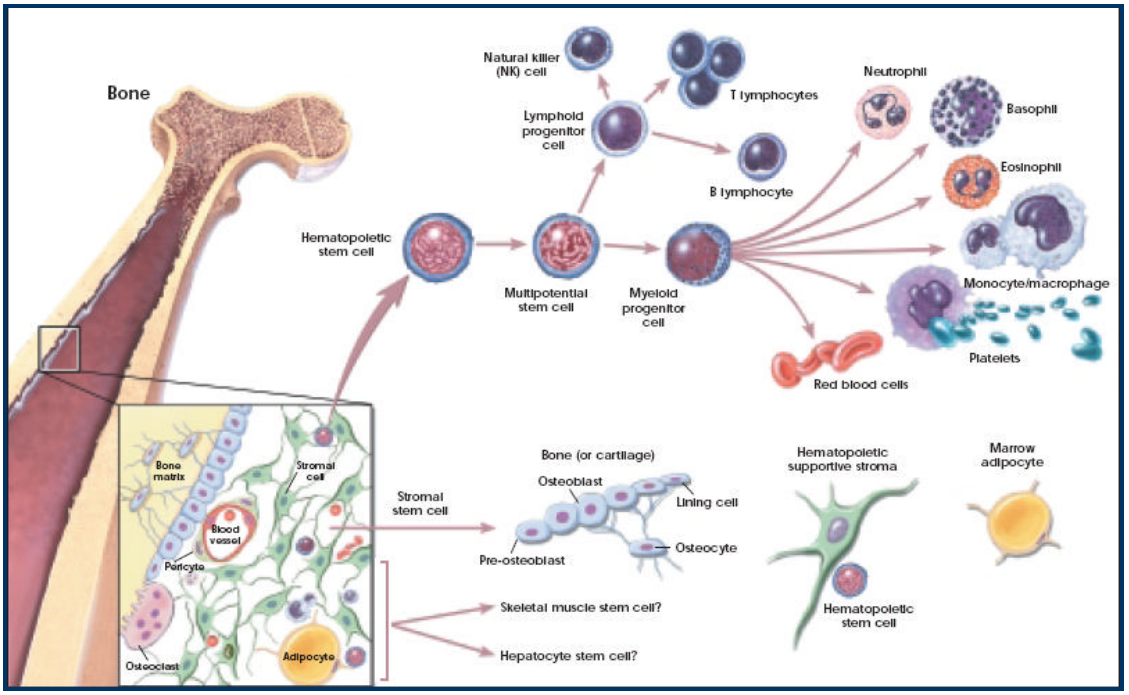


Figure 12. Hematopoietic and Stromal Stem Cell Differentiation.

Adult stem cells and cord blood stems cells have thus far been the only stem cells used to successfully treat any diseases. Diseases treated by these non-embryonic stem cells include a number of blood and immune-system related genetic diseases, cancers, and disorders; juvenile diabetes; Parkinson's; blindness and spinal cord injuries. Besides the ethical problems of stem cell therapy, there is a technical problem of graft-versus-host disease associated with allogeneic stem cell transplantation. However, these problems associated with histocompatibility may be solved using autologous donor adult stem cells or via therapeutic cloning.

Although differentiated beta cells are difficult to proliferate and culture, some researchers have had success in engineering such cells to do this. For

example, Fred Levine and his colleagues at the University of California, San Diego, have engineered islet cells isolated from human cadavers by adding to the cells' DNA special genes that stimulate cell proliferation. However, because once such cell lines that can proliferate in culture are established, they no longer produce insulin. The cell lines are further engineered to express the beta islet cell gene, PDX-1, which stimulates the expression of the insulin gene. Such cell lines have been shown to propagate in culture and can be induced to differentiate to cells, which produce insulin. When transplanted into immune-deficient mice, the cells secrete insulin in response to glucose. The researchers are currently investigating whether these cells will reverse diabetes in an experimental diabetes model in mice[121,122].

These investigators report that these cells do not produce as much insulin as normal islets, but it is within an order of magnitude. The major problem in dealing with these cells is maintaining the delicate balance between growth and differentiation. Cells that proliferate well do not produce insulin efficiently, and those that do produce insulin do not proliferate well. According to the researchers, the major issue is developing the technology to be able to grow large numbers of these cells that will reproducibly produce normal amounts of insulin.

Another promising source of islet progenitor cells lies in the cells that line the pancreatic ducts. Some researchers believe that multipotent (capable of forming cells from more than one germ layer) stem cells are intermingled with mature, differentiated duct cells, while others believe that the duct cells themselves can undergo a differentiation, or a reversal

to a less mature type of cell, which can then differentiate into an insulin-producing islet cell. Susan Bonner-Weir and her colleagues reported some years ago that when ductal cells isolated from adult human pancreatic tissue were cultured, they could be induced to differentiate into clusters that contained both ductal and endocrine cells. Over the course of three to four weeks in culture, the cells secreted low amounts of insulin when exposed to low concentrations of glucose, and higher amounts of insulin when exposed to higher glucose concentrations. The researchers have determined by immunochemistry and ultrastructural analysis that these clusters contain all of the endocrine cells of the islet[1 2 3].

According to the researchers, it might be possible in principle to do a biopsy and remove duct cells from a patient and then proliferate the cells in culture and give the patient back his or her own islets. This would work with patients who have T1D and who lack functioning beta cells, but their duct cells remain intact. However, the autoimmune destruction would still be a problem and potentially lead to destruction of these transplanted cells. T2D patients might benefit from the transplantation of cells expanded from their own duct cells since they would not need any immunosuppression. However, many researchers believe that if there is a genetic component to the death of beta cells, then beta cells derived from ductal cells of the same individual would also be susceptible to autoimmune attack.

Some researchers question whether the ductal cells are indeed undergoing a dedifferentiation or whether a subset of stem-like or islet progenitors populate the pancreatic ducts and may be co-cultured along with the

ductal cells. If ductal cells die off but islet precursors proliferate, it is possible that the islet precursor cells may overtake the ductal cells in culture and make it appear that the ductal cells are dedifferentiating into stem cells. According to Bonner-Weir, both dedifferentiated ductal cells and islet progenitor cells may occur in pancreatic ducts.

Ammon Peck of the University of Florida, Vijayakumar Ramiya of Ixion Biotechnology in Alachua, FL, and their colleagues have also cultured cells from the pancreatic ducts from both humans and mice. They reported that pancreatic ductal epithelial cells from adult mice could be cultured to yield islet-like structures similar to the cluster of cells found by Bonner-Weir. Using a host of islet-cell markers they identified cells that produced insulin, glucagon, somatostatin, and pancreatic polypeptide. When the cells were implanted into diabetic mice, the diabetes was reversed[124].

Joel Habener has also looked for islet-like stem cells from adult pancreatic tissue. He and his colleagues have discovered a population of stem-like cells within both the adult pancreas islets and pancreatic ducts. These cells do not express the marker typical of ductal cells, so they are unlikely to be ductal cells, according to Habener. Instead, they express a marker called nestin, which is typically found in developing neural cells. The nestin-positive cells do not express markers typically found in mature islet cells. However, depending upon the growth factors added, the cells can differentiate into different types of cells, including liver, neural, exocrine pancreas, and endocrine pancreas, judged by the markers they express, and can be maintained in culture for up to eight months[125].

Useful sources of adult stem cells are being found in organs all over the body and of particular interest for diabetes therapy are stem cells from cord blood[126,127], pancreas[128,129], liver[130] and adipose tissue[131]. Today there are different positions regarding the use of bone marrow derived stem cells to cure diabetes. Bone marrow stromal stem cells are known to be able to transform into different cells types and most importantly into insulin producing cells.

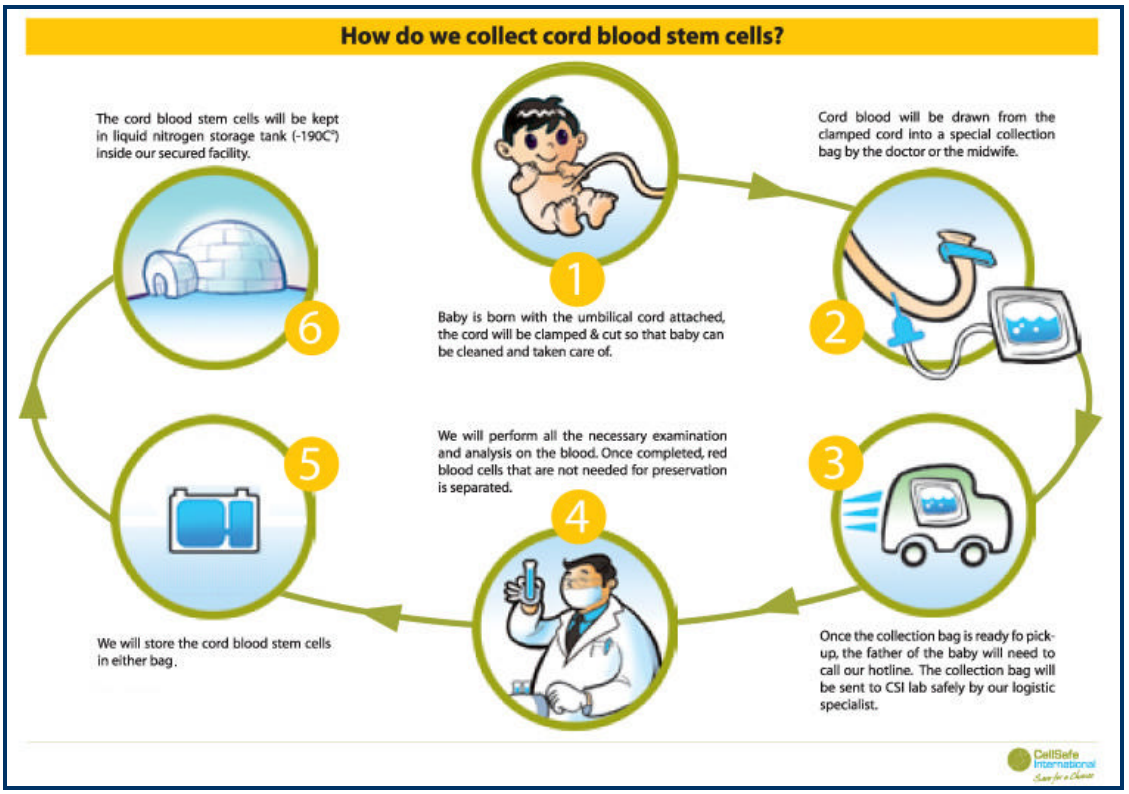


Figure 13. Collection of cord blood stem cells.

Blood from the placenta and umbilical cord that are left over after birth is one source of adult stem cells. Since 1988 these cord blood stem cells have been used to treat Gunther's disease, Hunter syndrome, Hurler syndrome, acute lymphocytic leukaemia and many more problems occurring mostly in children. It is collected by removing the umbilical cord, cleansing it and withdrawing blood from the umbilical vein.

Stem cells from the blood of newborns are more proliferate and have a higher chance of matching family members than stem cells from bone

marrow. Parents and siblings match 50% of the genetic markers of the donor's stem cell. However, since many different genetic markers are required for a match, the probability of a potential implant in a parent or sibling is considerably lower. These cells could be a good material from which to rebuild tissues and organs during adult life[132,133]. This potential outcome is the reason for which the set up of cord blood banks is based. In these banks this biological material can be stored from birth for possible future use (see figure 13).

4.4.2.2 ADIPOSE TISSUE DERIVED STEM CELLS

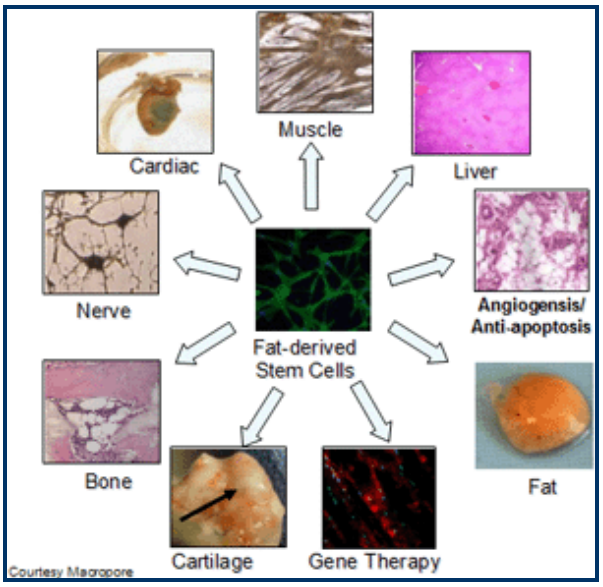


Figure 14. Human adipose tissue-derived stem cells differentiation.

Adipose tissue is an abundant, accessible and replenishable source of adult stem cells that can be isolated from liposuction waste tissue by collagenase digestion and differential centrifugation.

Adipose tissue derived stem cells are multipotent, differentiating along the adipocyte, chondrocyte, myocyte, neuronal and osteoblast lineages (see figure 14).

This cell population seems to be similar in many ways to mesenchymal stem cells (MSCs) derived from bone marrow. However, it is possible to isolate many more cells from adipose tissue and the harvest procedure itself is less painful than the harvest of bone marrow. Human ASCs have

been shown to differentiate in the lab into bone, cartilage, fat, and muscle, while ASCs from rats have been converted to neurons, which makes ASCs a possible source for future applications in the clinic[134,135]. In support of this, current studies in animals suggest that ASCs might be able to repair significant bony defects and ASCs have been recently used to successfully repair a large cranial defect in a human patient.

4.4.2.3 BONE MARROW DERIVED STEM CELLS

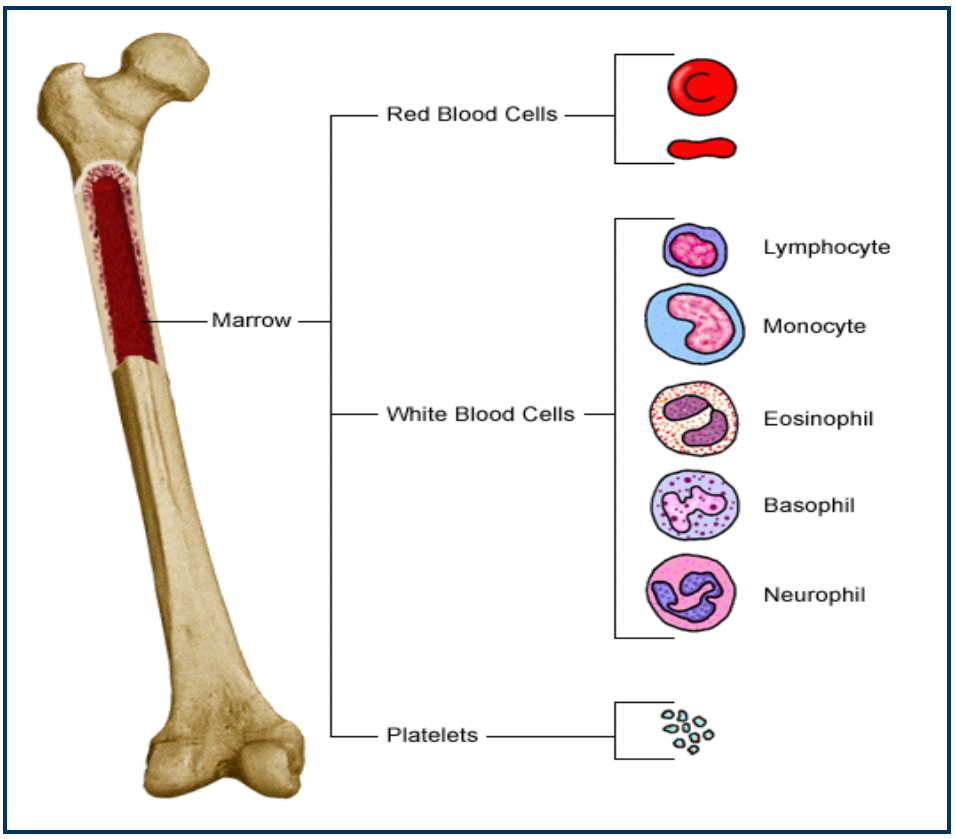


Figure 15. Bone marrow derived stem cells.

The notion that the bone marrow contains stem cells is not new. One population of bone marrow cells, the HSCs, is responsible for forming all

of the types of blood cells in the body (see figure 15). HSCs were recognized as a stem cells more than 40 years ago. Bone marrow stromal cells, a mixed cell population that generates bone, cartilage, fat, fibrous connective tissue, and the reticular network that supports blood cell formation, were described shortly after the discovery of HSCs[136]. The mesenchymal stem cells of the bone marrow also give rise to these tissues, and may constitute the same population of cells as the bone marrow stromal cells. Of all the cell types in the body, those that survive for the shortest period of time are blood cells and certain kinds of epithelial cells. For example, red blood cells (erythrocytes), which lack a nucleus, live for approximately 120 days in the bloodstream. The life of an animal literally depends on the ability of these and other blood cells to be replenished continuously. This replenishment process occurs largely in the bone marrow, where HSCs reside, divide, and differentiate into all the blood cell types.

HSCs can reconstitute the hematopoietic system of mice that have been subjected to lethal doses of radiation to destroy their own hematopoietic systems. This test, the rescue of lethally irradiated mice, has become a standard by which other candidate stem cells are measured because it shows, without question, that HSCs can regenerate an entire tissue system, in this case, the blood. HSCs were first proven to be blood-forming stem cells in a series of experiments in mice; similar blood-forming stem cells occur in humans. HSCs are defined by their ability to self-renew and to give rise to all the kinds of blood cells in the body. This means that a single HSC is capable of regenerating the entire hematopoietic system, although this has been demonstrated only a few times in mice. Over the years, many

combinations of surface markers have been used to identify, isolate, and purify HSCs derived from bone marrow and blood.

Undifferentiated HSCs and hematopoietic progenitor cells express c-kit, CD34, and H-2K. Two kinds of HSCs have been defined. Long-term HSCs proliferate for the lifetime of an animal. In young adult mice, an estimated 8 to 10 % of long-term HSCs enter the cell cycle and divide each day. Short-term HSCs proliferate for a limited time, possibly a few months. Long-term HSCs have high levels of telomerase activity.

In adult humans, HSCs occur in the bone marrow, blood, liver, and spleen, but are extremely rare in any of these tissues. In mice, only 1 in 10,000 to 15,000 bone marrow cells is a long-term HSC.

Short-term HSCs differentiate into lymphoid and myeloid precursors, the two classes of precursors for the two major lineages of blood cells. Lymphoid precursors differentiate into T cells, B cells, and natural killer cells. The mechanisms and pathways that lead to their differentiation are still being investigated[137,138]. Myeloid precursors differentiate into monocytes and macrophages, neutrophils, eosinophils, basophils, megakaryocytes, and erythrocytes[139]. In vivo, bone marrow HSCs differentiate into mature, specialized blood cells that cycle constantly from the bone marrow to the blood, and back to the bone marrow. A recent study showed that short-term HSCs are a heterogeneous population that differ significantly in terms of their ability to self-renew and repopulate the hematopoietic system[140].

Many of the soluble factors that regulate HSC differentiation in vivo are cytokines, which are made by different cell types and are then

concentrated in the bone marrow by the extracellular matrix of stromal cells, the sites of blood formation. Two of the most-studied cytokines are granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3).

Also important to HSCs proliferation and differentiation are interactions of the cells with adhesion molecules in the extracellular matrix of the bone marrow stroma.

Bone marrow (BM) stromal cells have long been recognized for playing an important role in the differentiation of mature blood cells from HSCs. But stromal cells also have other important functions. In addition to providing the physical environment in which HSCs differentiate, BM stromal cells generate cartilage, bone, and fat.

BM stromal cells have many features that distinguish them from HSCs. The two cell types are easy to separate in vitro. When bone marrow is dissociated, and the mixture of cells it contains is plated at low density, the stromal cells adhere to the surface of the culture dish, and the HSCs do not. Given specific in vitro conditions, BM stromal cells form colonies from a single cell called the colony forming unit-F (CFU-F). These colonies may then differentiate as adipocytes or myelosupportive stroma, a clonal assay that indicates the stem cell-like nature of stromal cells. Unlike HSCs, which do not divide in vitro (or proliferate only to a limited extent), BM stromal cells can proliferate for up to 35 population doublings in vitro[141]. They grow rapidly under the influence of such mitogens as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1).

Panels of markers used to identify the cells include receptors for certain cytokines (interleukin-1, 3, 4, 6, and 7) receptors for proteins in the extracellular matrix, (ICAM-1 and 2, VCAM-1, the alpha-1, 2, and 3 integrins, and the beta-1, 2, 3 and 4 integrins), etc. Despite the use of these markers and another stromal cell marker called Stro-1, the origin and specific identity of stromal cells have remained elusive. Like HSCs, BM stromal cells arise from embryonic mesoderm during development, although no specific precursor or stem cell for stromal cells has been isolated and identified. One theory about their origin is that a common kind of progenitor cell, perhaps a primordial endothelial cell that lines embryonic blood vessels, gives rise to both HSCs and to mesodermal precursors.

4.5 STEM CELL PLASTICITY

By definition, stem cells are capable of both self-renewal and differentiation into at least one mature cell type. Stem cells are subclassified based on their species of origin, tissue of origin, and potential to differentiate into one or more specific types of mature cells. Some stem cells are more pluripotent than others. Thus, a fertilized egg is totipotent and an adult (postnatal) stem cells, although still pluripotent, have been thought to have even more limited differentiation ability and to be organ specific.

Traditionally, adult stem cells have been viewed as committed to a particular cell fate to produce cells from the tissue of origin but not cells of non related tissues. Various reports over the last six years challenge this

central dogma by demonstrating that adult stem cells, under certain micro environmental conditions, give rise to cell types besides the cell type in the tissue of origin possibly indicating that they can switch cell fate .

There are some general criteria to define stem cell plasticity:

- A single cell differentiate into multiple cell lineages.
- Differentiated cells are functional in vitro and in vivo.
- Engraftment is robust and persistent.

The best studied adult stem cell, the haematopoietic stem cell (HSC), resides in adult life in the bone marrow (BM)[142]. Presence of HSC is demonstrated by the ability of transplanted donor cells to reconstitute the hematopoietic system of a lethally myeloablated host. Murine HSC reside in the lineage negative fraction of cells, and express the stem cell antigens Sca-1, and low levels of c-Kit and Thy-1. In humans, HSC are also lineage negative and further enriched in the CD34+CD38- subpopulation.

Aside from HSC, several other stem cells exist in bone marrow. These include mesenchymal stem cells (MSC). MSC can be isolated from BM[143] as well as adipose tissue[144] and fetal lung[145]. They lack the hematopoeitic surface marker, CD45, but in humans express CD105 (SH2), SH3, Stro-1 and CD13. The other rare cell type that can be culture isolated from BM and other organs (brain, muscle) is termed multipotent adult progenitor cell or MAPC[146].

Many of the findings in this new field are controversial, in part because (i) few of the techniques used thus far to assess in vitro and in vivo plasticity

are convincing, (ii) existing paradigms of cellular differentiation do not yet include postnatal switching of cell fate, and (iii) it is unclear how this phenomenon can be safely and reasonably exploited for therapeutic use in humans.

To be able to claim that adult stem cells demonstrate plasticity, it is first important to show that a cell population exists in the starting tissue that has the identifying features of stem cells. Then, it is necessary to show that the adult stem cells give rise to cell types that normally occur in a different tissue. Neither of these criteria is easily met. Simply proving the existence of an adult stem cell population in a differentiated tissue is a laborious process. It requires that the candidate stem cells are shown to be self-renewing, and that they can give rise to the differentiated cell types that are characteristic of that tissue. To show that the adult stem cells can generate other cell types requires them to be tracked in their new environment, whether it is in vitro or in vivo. In general, this has been accomplished by obtaining the stem cells from a mouse that has been genetically engineered to express a molecular tag in all its cells. It is then necessary to show that the labeled adult stem cells have adopted key structural and biochemical characteristics of the new tissue they are claimed to have generated. Ultimately, and most importantly, it is necessary to demonstrate that the cells can integrate into their new tissue environment, survive in the tissue, and function like the mature cells of the tissue.

In the experiments reported to date, adult stem cells may assume the characteristics of cells that have developed from the same primary germ

layer or a different germ layer. For example, many plasticity experiments involve stem cells derived from bone marrow, which is a mesodermal derivative. The bone marrow stem cells may then differentiate into another mesodermally derived tissue such as skeletal muscle, cardiac muscle or liver.

Alternatively, adult stem cells may differentiate into a tissue that, during normal embryonic development, would arise from a different germ layer. For example, bone marrow-derived cells may differentiate into neural tissue, which is derived from embryonic ectoderm. And, reciprocally, neural stem cell lines cultured from adult brain tissue may differentiate to form hematopoietic cells, or even give rise to many different cell types in a chimeric embryo. In both cases cited above, the cells would be deemed to show plasticity, but in the case of bone marrow stem cells generating brain cells, the finding is less predictable.

In order to study plasticity within and across germ layer lines, the researcher must be sure that is using only one kind of adult stem cell. The vast majority of experiments on plasticity have been conducted with adult stem cells derived either from the bone marrow or the brain. The bone marrow-derived cells are sometimes sorted, using a panel of surface markers, into populations of hematopoietic stem cells or bone marrow stromal cells[147,148,149]. The HSCs may be highly purified or partially purified, depending on the conditions used. Another way to separate population of bone marrow cells is by fractionation to yield cells that adhere to a growth substrate (stromal cells) or do not adhere (hematopoietic cells).

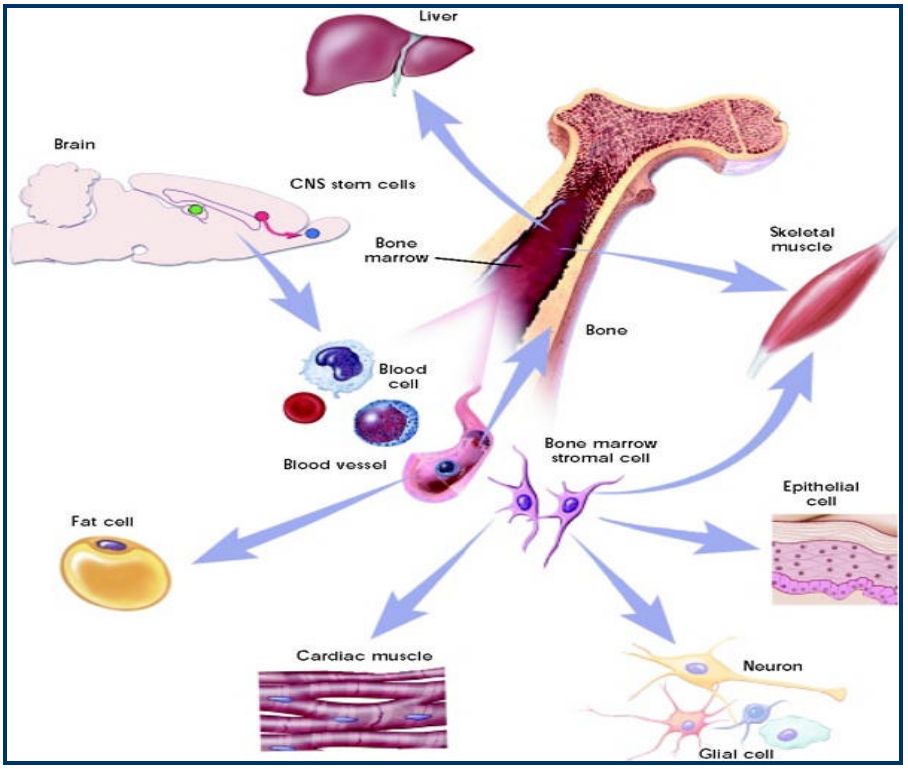


Figure 16. Evidence of Plasticity Among Nonhuman Adult Stem Cells.

4.5.1 EVIDENCE FOR STEM CELLS PLASTICITY

The differentiated cell types that result from plasticity are usually reported to have the morphological characteristics of the differentiated cells and to display their characteristic surface markers. In reports that transplanted adult stem cells show plasticity in vivo, the stem cells typically are shown to have integrated into a mature host tissue and assumed at least some of its characteristics. Many plasticity experiments involve injury to a particular tissue, which is intended to model a particular human disease or injury. However, there is limited evidence to date that such adult stem cells can generate mature, fully functional cells or that the cells have

restored lost function in vivo. Collectively, studies on plasticity suggest that stem cell populations in adult mammals are not fixed entities, and that after exposure to a new environment, they may be able to populate other tissues and possibly differentiate into other cell types. It is not yet possible to say whether plasticity occurs normally in vivo. Also, it is not yet clear to what extent plasticity can occur in experimental settings, and how the phenomenon can be harnessed to generate tissues that may be useful for therapeutic transplantation. If the phenomenon of plasticity is to be used as a basis for generating tissue for transplantation, the techniques for doing it will need to be reproducible and reliable. In some cases, debate continues about observations that adult stem cells yield cells of tissue types different than those from which they were obtained.

4.6 AIM OF THE STUDY

The aim of this long term project is to investigate the effect of stem cells transplantation of bone marrow derived stem cells from non diabetic NOD mice to diabetic NOD mice. The rationale behind this choice of experiment is that stem cells could regenerate pancreatic beta cells to resume the production of insulin (see Figure 17).

The project is divided in three phases:

- **First phase:** to establish the best conditions for the isolation and cultivation of mesenchymal stem cells from NOD mice, which represent

one of the ideal sources that can be used for the generation of new insulin producing cells.

- Second phase: to focus on the proliferation and differentiation of these cells to generate new insulin producing cells.

- Third phase: to inject these new generated insulin producing cells in NOD mice to see if we can delay or prevent the development of the disease.

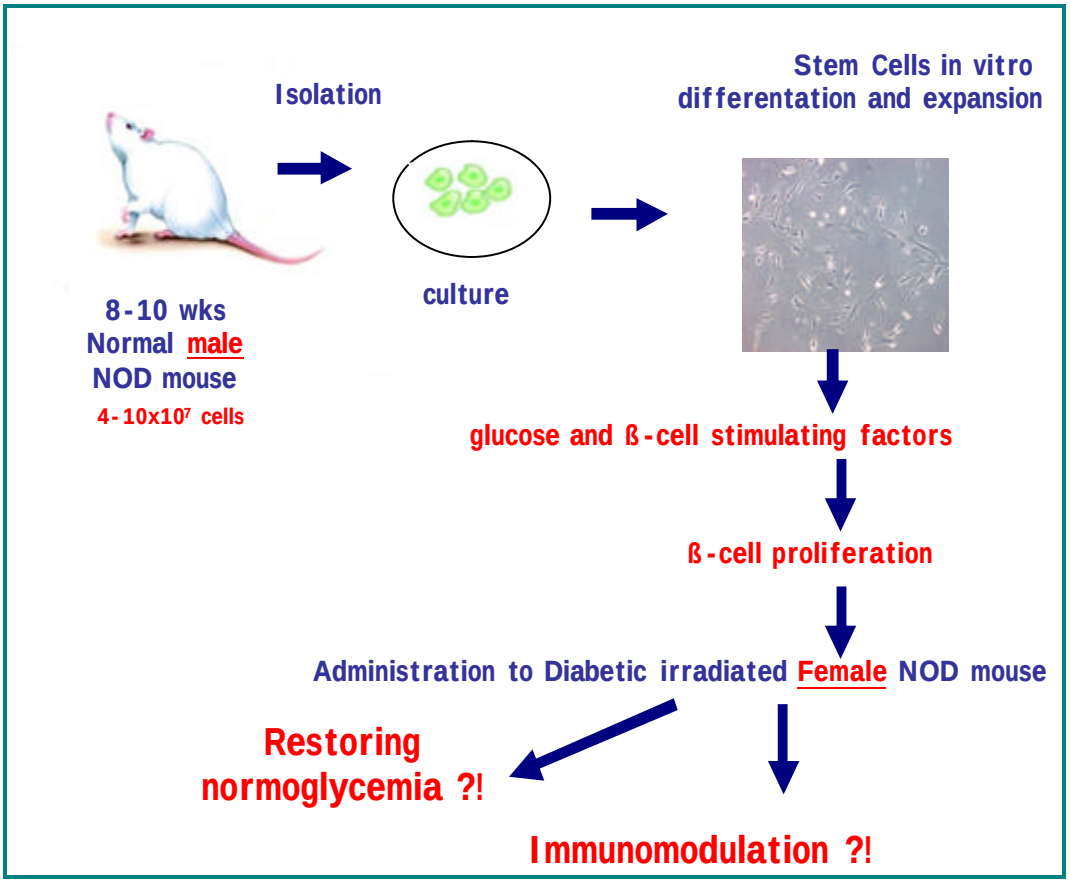


Figure 17. Plan of work.

4.7 MATERIALS AND METHODS

We isolated mesenchymal stem cells (MSCs) from the bone marrow of NOD mice according to a protocol modified from Dobson et al[150].

1o NOD mice were killed by cervical dislocation according to UK Home Office regulations and the tibiae and femurae aseptically removed. Bone marrow cells (BMC) were recovered using centrifugation procedure. Proximal ends were removed, bones were then placed in microfuge tubes supported by plastic inserts cut from 0.5 ml microfuge tube or hypodermic needle casings and briefly centrifuged at 2000 rpm for 5 seconds. The marrow pellet was resuspended in 10 ml culture medium. A single cell suspension was achieved by passing through a 21 gauge needleand and cells were counted using a haemocytometer.

Pelleted bone marrow cells were plated in a-MEM medium supplemented with vitamin C, 10% fetal bovine serum and 1x antibiotics-antimycotics.

Cells were left overnight and then washed to remove any non-adherent cells. The remaining adherent cells were cultured for 2 weeks to allow colony formation. From a single bone we obtained about 100,000 cells and we believe that this is a good demonstration of the efficiency of the method that we used.

To confirm isolation of MSCs we used three different mesenchymal stem cells markers including STRO-1, VCAM-1 (CD106) and Endoglin (CD105). Immunocytochemistry was carried out using specific monoclonal antibodies to these MSC cell surface markers, a secondary antibody

conjugated to FITC was used and when analysed on a fluorescent microscope positive staining for STRO-1, VCAM-1 (CD106), Endoglin (CD105) and Vimentin could clearly be seen (in the majority of cells), demonstrating that the cells are mesenchymal stem cells.

- ***STRO-1*** : This is a surface antigen expressed by stromal cell precursors in bone marrow. The subset of bone marrow cells that express the STRO-1 antigen is capable of differentiating into multiple mesenchymal lineages[151].
- ***VCAM-1 (CD106)*** : It is a vascular cell adhesion molecule and expressed by mesenchymal stem cells[152].
- ***ENDOGLIN (CD105)*** : Endoglin is a homodimeric type1 transmembrane glycoprotein. It has been shown that multipotent stem cells can be isolated from bone marrow based on their expression of Endoglin[153,154]. Endoglin was also shown to be a functional marker that defines long-term repopulating hematopoetic stem cells from bone marrow side-population cells[155].

As a positive control we used Vimentin an intermediate filament protein that forms part of the cytoskeleton of vertebrate cells. It labels cells of mesenchymal origin.

As negative control Keratin1 was used which is not expressed in stem cells.

4.8 RESULTS

Positive cells were detected by indirect immunofluorescence using FITC (Fluorescein Isothiocyanate) labelled antibodies to the above mentioned

markers and visualized using fluorescent microscope. For STRO-1and for the positive and negative controls we got good results (see Figures 18-20). The staining for VCAM-1 and Endoglin was also seen but it wasn't so good and now we are working to optimise the conditions.

Results showed that bone marrow stem cells from NOD mice can acquire the capacity of expressing markers of MSCs and it suggests that these cells are indeed MSCs.

We also analysed these cells with Fluorescence Activated Cell Sorter (FACS) to confirm that they were MSCs. The Endoglin FACS showed 41% positive cells (see Figure 21).

These results are very interesting also considering that they will provide an unlimited source of autologous stem cells, which can be transformed into insulin producing cells.

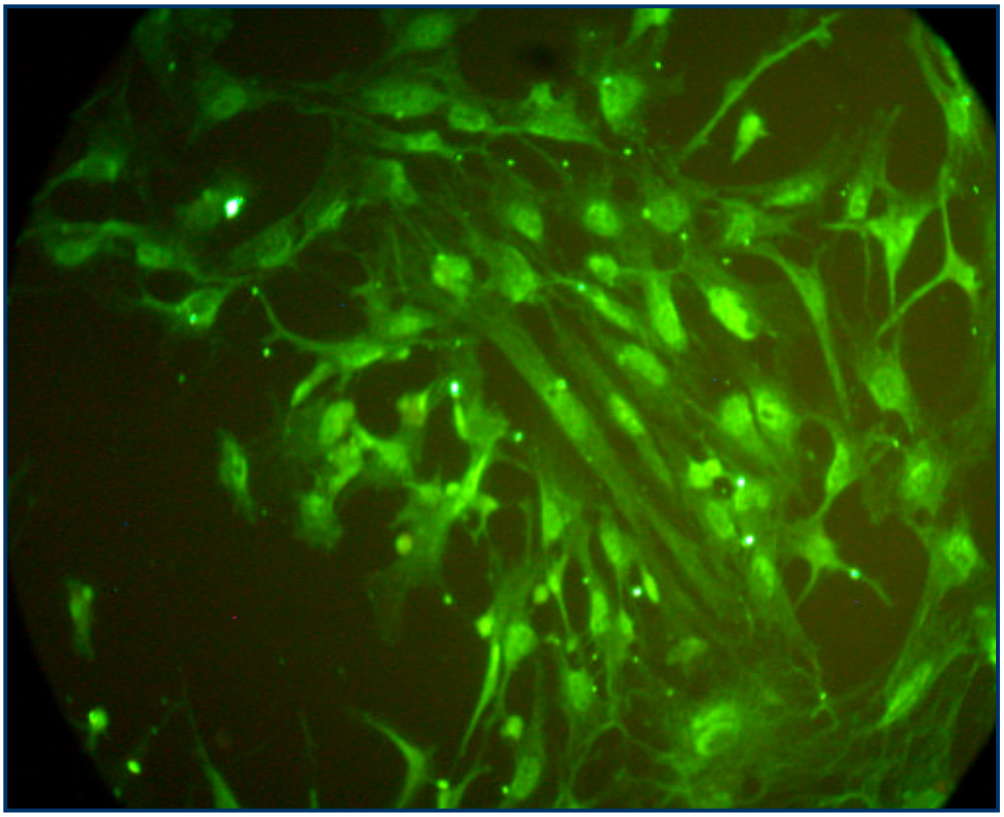


Figure 18. Staining for STRO-1 (magnification X40)

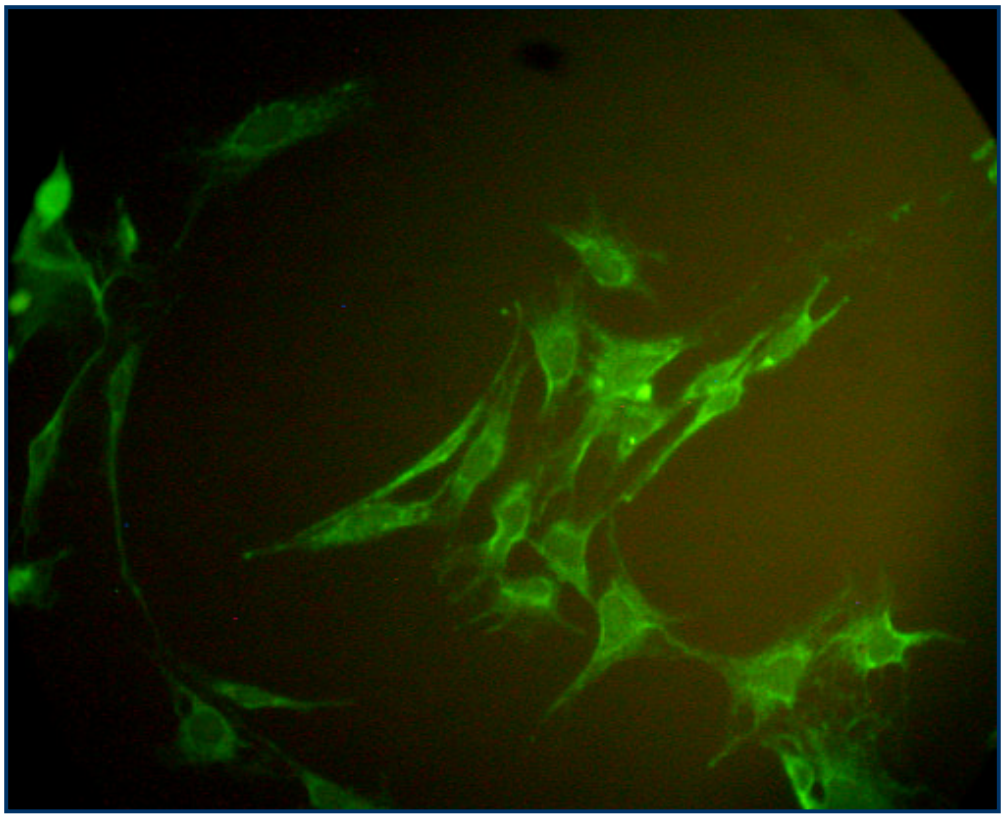


Figure 19. Staining for Vimentin –positive control- (magnification X40)

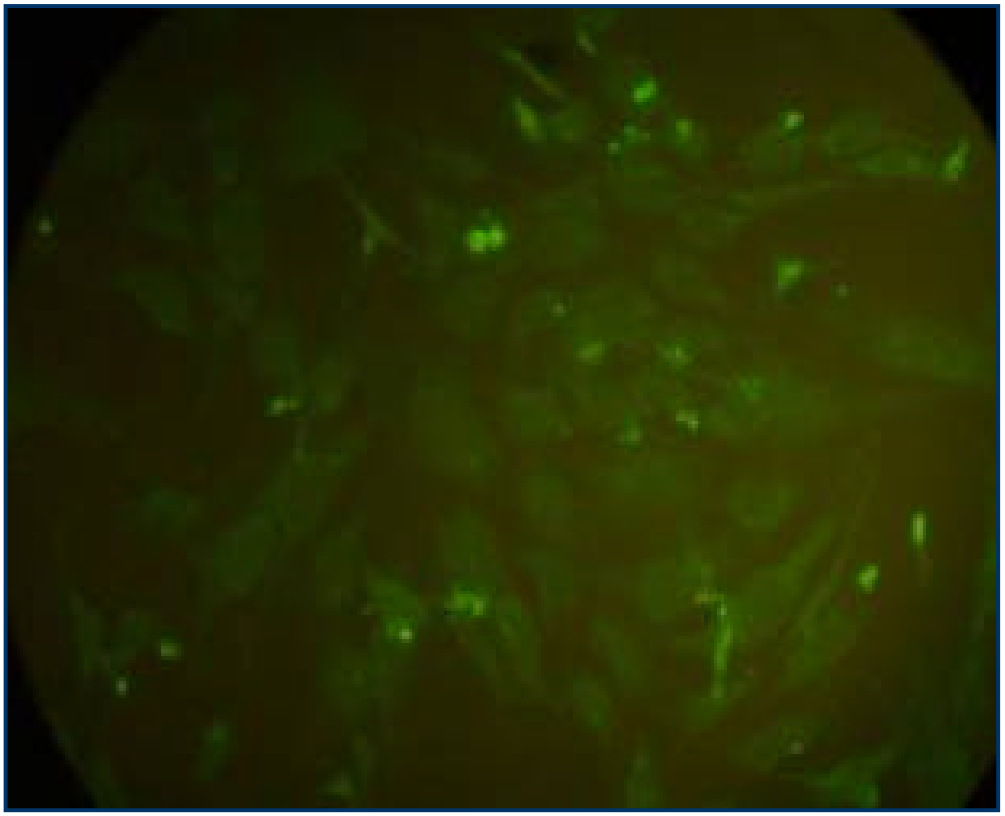


Figure 20. Negative control (magnification X40)

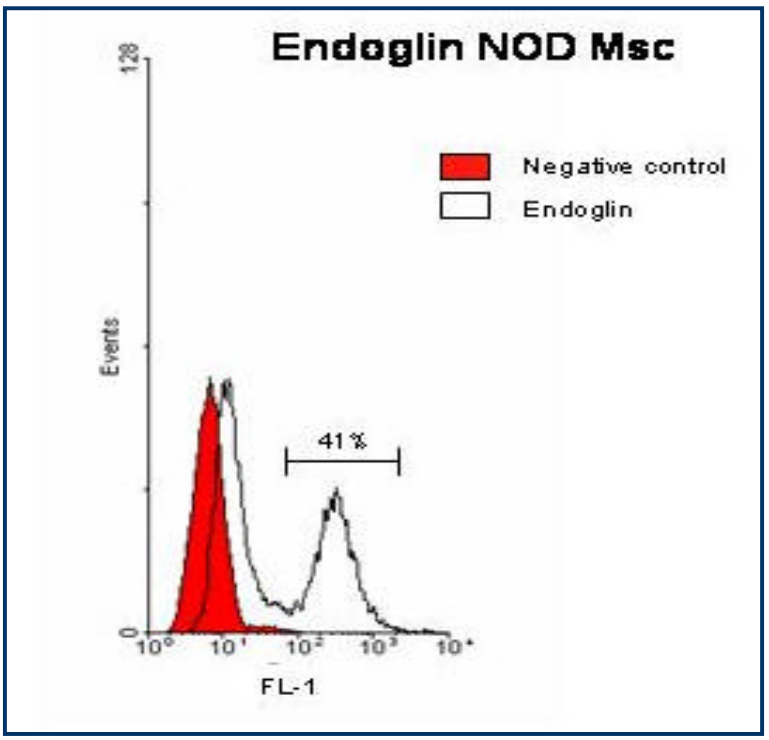


Figure 21. FACS Analysis

4.9 DISCUSSION

T1D is one of the more costly chronic diseases of children and adolescents throughout North America and Europe, exhibiting an average estimated prevalence rate of nearly 0.2%. While routine insulin therapy can provide diabetic patients with their daily insulin requirements, non-compliance and undetected hyperglycemic excursions often lead to subsequent long-term microvascular and macrovascular complications. Today the only real cure for T1D is replacement of the beta cell mass, currently being accomplished through ecto-pancreatic transplantation and islet implantation.

Three alternative approaches are: (i) the production of surrogate cells by genetically modifying non-endocrine cells to secrete insulin in response to glucose challenge; (ii) the trans-differentiation of non-endocrine stem/progenitor cells or mature cells to glucose-responsive adult tissue and (iii) the regulated differentiation of islet stem/progenitor cells to produce large numbers of mature, functional islets. In recent years, each of these approaches has made impressive advances, leading to the most important question, "how soon will this new science be available to the patient?"

Stem cell therapy and stem cell research have been a hot topic over the last several years, giving rise to much debate, however, there are too many pros and cons to stem cell research and stem cell therapy.

The pros of stem cell research have led to many therapies now available to treat a range of diseases and disorders, from various types of cancer to spinal cord injuries. Stem cell research has shown that stem cells have an amazing ability to regenerate all areas of the body, repairing damaged or diseased tissues and curing such degenerative diseases as Alzheimer's disease.

The pros of stem cell research all center around the use of adult stem cells, neural stem cells and cord blood stem cells. There are no moral or ethical questions about the use of these types of stem cells. Those who are pro stem cell research believe that researching and using these stem cells can only lead to progress and medical discoveries that will help us all live longer and healthier lives.

The cons of stem cell research mostly refer to issues with embryonic stem cell research. Those who oppose stem cell research oppose the use of embryonic stem cells. The cons of stem cell research using embryonic stem cells surround the question of whether or not a human life is being lost when the embryonic stem cells are extracted. To use these cells for research, the embryo must be destroyed and this is an ethical issue.

Considering all these troubles and concerns we slowed down our project on stem cells and we focused on the regenerative capability of the beta cell mass and on the immunomodulatory effects of MSCs.

These cells, infact, have the ability to differentiate into multiple cell types and this potential raises exciting therapeutic possibilities. A recent report described the successful use of MSCs for the treatment of graft-versus-host

disease; however, the scientific community has yet to define the molecular mechanisms of immunomodulation by MSCs[156].

Beta cell regeneration has quickly become one of the more promising cure therapeutic areas in diabetes, even though this field barely existed five years ago. Members of the JDRF's Regeneration of Beta Cell Function program team have screened a library of approximately 100,000 clinical compounds that might expand beta cells.

Basic discoveries by team members also advanced the field. Markus Stoffel of New York's Rockefeller University identified a protein called Tmem27 that regulates cell growth in pancreatic islets, but not in other cell types[157]. This discovery provides an entry point for developing therapies to stimulate beta cell growth without causing harmful side effects in other healthy tissues. And at Tel Aviv University in Israel, Shimon Efrat successfully expanded human islets in culture[158]. Efforts are now focused on restoring the cells' functionality to see if they can be exploited in developing regeneration therapeutics.

CHAPTER 5: REG GENES AND BETA CELLS REGENERATION

5.1 BACKGROUND

Increases in beta cell mass may occur through increased beta cell replication, increased beta cell size, decreased beta cell death, and differentiation of possibly existing beta cell progenitors[159].It has been shown that occasional endocrine cells can be found embedded in normal pancreatic ducts[160]. The number of these duct-associated endocrine cells physiologically increases as the consequence of severe insulin resistance in obese individuals or during pregnancy [161,162]. Similar histological changes are observed under conditions of tissue injury and repair after partial pancreatectomy, duct ligation, cellophane wrapping of the gland, or IFN-? over expression driven by the insulin promoter[163,164,165,166].

Even then, within the ducts, only a small number of cells become insulin positive. This suggests that even if some hypothetical precursors exist, the process of formation of endocrine cells out of the islet (neogenesis) would not be a frequently observed property of the duct epithelium. On the other hand, the fact that a and beta cells develop from a possibly common, non hormone-expressing, yet Pdx1 positive precursor (Pdx1 being a transcription factor required for pancreatic development) suggests that all cell types found within the islet may originate from a common endocrine progenitor[167].

These endocrine progenitors may be located close to the duct but may not actually be components of the ductal epithelium[168]. The progenitor cells could be mesenchymal in origin, or they could be cells differentiated from

an unknown cell type. If the number of these progenitors is extremely small, lineage analysis becomes very difficult because of the lack of known appropriate markers. Moreover, if these cells are as rare as they appear to be, it becomes difficult to quantify their contribution to normal endocrine cell turnover.

These are some of the conclusions discussed by Weir and Bonner-Weir[169] in commenting on the study by Seaberg et al, in which it was shown that single murine adult pancreatic precursor cells can generate progeny with characteristics of pancreatic cells, including beta cells. These rare (1 in 3,000-9,000 cells) pancreas derived multipotent precursors (PMPs) do not seem to be pluripotent ES cells; nor are these cells of clear ectodermal, mesodermal, or endodermal origin, since they failed to express other markers considered specific for precursors of each of the embryonic cell types. Because, surprisingly, these PMPs also lacked some beta cell markers (e.g.,HNF3 beta) as well as ductal epithelium markers (e.g., cytokeratin), but were able to generate differentiation products with neural characteristics along with alpha, beta, delta and acinar pancreatic cells, the authors proposed the existence of a new and unique ectodermal/endodermal precursor cell present during embryonic development that could persist in adult tissues[170].

These results support the conclusions of another recent study in which multipotent pancreatic progenitors were prospectively isolated using flow cytometric cell sorting[171]. The marker used in this case was c-Met, the HGF receptor. The rationale for this choice was the known signal exchange between epithelial and mesenchymal cells, promoting the interaction

between c-Met and HGF, which plays an important role in the development of the pancreas. The authors suggest that c-Met/HGF interaction is critically responsible for growth and differentiation of pancreatic stem and progenitor cells not only during development but also in the adult, where they maintain homeostasis and promote regeneration. Colonies derived from single c-Met positive cells, sorted from neonatal and adult mouse pancreatic tissues, contained cells expressing several markers for endocrine, acinar and ductal lineage cells.

All of these studies, even with their somewhat divergent outcomes, seem to support the conclusion that endocrine precursor cells of some kind exist in the pancreas. They are present not only in the duct, but also within the islets themselves, since both subpopulations were independently used as the source of the isolated single cell precursors. On one hand, this conclusion supports the working hypothesis of those who propose that pancreatic ductal cells can transdifferentiate into beta cells and that this is a physiologic process generally more efficiently activated by increased metabolic demand and tissue injuries[172]; on the other hand, it may also accommodate the most recent results of Dor and colleagues[173], who propose instead that no beta cell can arise from non beta cell progenitors, whether in the normal adult pancreas or after pancreatectomy. As a direct consequence, the number of beta cells should become virtually defined at a certain point, and, afterward, glycaemia should be controlled only by that defined cellular pool. Dor's results were obtained by using a sophisticated Cre/lox system that, in transgenic mice, can be induced by tamoxifen. This system labels fully differentiated beta cells (defined as postnatal cells

transcribing the insulin gene) that express the human alkaline phosphatase protein, which is in turn revealed by a histochemical stain. In a defined period of time, the "chase", only the cells that are progeny of pre-existing and labelled beta cells are newly labelled. New beta cells derived from any non beta cell source, including stem cells, are not labelled. The frequency and distribution of labelled beta cells within pancreatic islets, at the end of the chase period, should be inversely proportional to the number of new, non labelled cells present in the same structures. If the frequency of labelled beta cells does not change, as was observed, the number of cells derived from the differentiation of non insulin-producing precursors must be minimal or null, while terminally differentiated insulin-producing beta cells themselves should be the cells that actually proliferate and give rise to other insulin-producing beta cells. While the results of Seaberg et al. do not contest the proven yet limited ability of a beta cell to divide, the failure of Dor et al. to observe cells possibly differentiated from stem or precursor cells might actually be due to both their extremely limited number and technical issues[174].

5.2 REG GENES

In humans and in most animals, pancreatic beta cells are the only cells that produce insulin. These cells are destroyed by the autoimmune process that leads to T1D. There is evidence, however, that the beta cell mass is not static: it is in a constant process of death and renewal (regeneration/self-duplication). The limited capacity for regeneration is a predisposing factor for the development of diabetes. Strategies for influencing the replication

and growth of the beta cell mass are therefore important for the prevention and/or treatment of diabetes.

In 1984, it was found that administration of poly(ADP-ribose) synthetase inhibitors such as nicotinamide to 90% depancreatized rats induced regeneration of pancreatic islets. In screening the regenerating islet-derived cDNA library, a novel gene, REG (i.e. regenerating gene), which is expressed in regenerating islets but not in the normal islets, insulinomas, or regenerating liver, was discovered[175].

Further studies isolated several REG and REG-related genes from human, rat and mouse, and revealed that they constitute a multigene family, the Reg gene family. Based on the amino-acid sequence homology among proteins encoded by Reg genes, the members of the family can be grouped into four subclasses, type I, II, III and IV.

In humans, the Reg family genes are ordered in the 95-kbp DNA region of chromosome 2p12[176]. Chromosomal organization of human REG genes is as follows: HIP/PAP -REG 1alpha –REG 1beta –PAP IB . The same chromosomal organization was found in the rat, where the genes encoding the three PAPs and the Reg proteins co-localize at 4q33-q34[177]. Mouse reg genes instead, reg 1, reg 2, reg 3alpha, reg 3beta, reg 3gamma, and reg 3delta[178] were assigned to chromosome 6[179]. They are disposed in the following order: 5'-reg 3beta -reg 3delta -reg 3alpha -reg 2 -reg 1-reg 3gamma-3'[180]. These results suggest that the REG family genes are derived from a common ancestor gene by several gene duplications, and have reached divergence in expression and function, depending on the different types of Reg genes, in the process of genetic evolution.

5.3 REG GENE FAMILY

Human **REG1alpha** gene maps to the short arm of chromosome 2 near the centromere at band 2p12[181]. It spans -3.0 kb, encodes a 166-amino acid protein including a 22-amino acid signal peptide and its molecular weight is 19 kDa.

It is expressed in high levels in fetal and infant brain. In adults, it is expressed, in low levels, also in brain. In addition, REG1alpha is expressed in Langerhans islets during beta cell regeneration; increased serum levels were observed in acute pancreatitis and chronic renal failure. Higher levels of urinary REG1alpha were found in patients with diabetic nephropathy. Together with HIP/PAP, REG1alpha is coexpressed in intestinal Paneth cells and in the brain of Alzheimer patients. The overexpression of REG1alpha is associated with several cancer diseases, e.g. pancreatic cancer derived from acinar or ductal cells, cholangiocarcinomas, cancer derived from colon, gastric and enterochromatin-like cells.

Generally, REG1alpha may act as mitogenic, antiapoptotic or anti-inflammatory factor; it can promote bacterial aggregation or increase resistance to antitumoral agents[182].

REG1alpha may be a sensitive marker for mucosa at risk for the development of neoplasia; increased secretion may reflect renal tubular dysfunction[183].

Human **REG 1beta** gene displays another pattern of expression: it is found in pancreas, colon and total brain in the fetus; in pancreas, jejunum, colon and pituitary gland in the adult[184].

Human **REG 3,** HIP/PAP (human counterpart of mouse REG 3beta) is expressed in normal Paneth cells, pancreas and hepatocellular carcinomas[185]. HIP/PAP has a possible involvement in antiapoptosis in a pancreatic acinar cell line, AR4-2J cells[186]. HIP and PAP are simply different names for a single protein derived from the same gene[187].

INGAP (islet neogenesis-associated protein)[188] may be a hamster homologue of type III REG. In the rat, the HIP/PAP gene shows a comparable tissue-specific expression pattern. It is overexpressed in the pancreas during acute pancreatitis, and also highly expressed in the columnar epithelial cells of ileum, jejunum and duodenum[189].

This protein from the REG family contains a putative IL-6 response element: HIP/PAP becomes over expressed in human diabetic islets because of the local inflammatory response.

Human **REG 3gamma** gene, also known as PAP IB, is expressed almost only in the pancreas[190]. A faint expression was observed in the placenta and PAP IB is absent in the small intestine. The peculiarity of this protein is its high homology with REG 1alpha: 50% sequence identity between the two proteins. The common protein fold and the sequence identity explain why these two proteins also share the same specific functions[191].

Human **REG 3delta** was not detected in normal islets but mainly expressed in exocrine pancreas. Although the other type III REG genes are expressed in the gastrointestinal tract, REG 3delta is not[192].

Human **REG 4** gene is the only Reg gene located on a different chromosome: chr1, 1p13.1-p12. This gene, also named RELP, is involved in inflammatory and metaplastic responses of the gastrointestinal epithelium and is up-regulated in malignancies of the human gastrointestinal tract[193]. REG 4 in fact is a potent activator of the EGF receptor/Akt/AP-1 signaling pathway in colorectal carcinoma and overexpression of REG 4 may be an early event in colorectal carcinogenesis[194].

Overexpression of REG 4 is also associated with pancreatic cancer and with hormone refractory metastatic prostate cancer.

Type	*Human*	*Rat*	*Mouse*	*Hamster*	*Cow*
I	Reg Ia Reg Iß	Reg I	Reg I		
II	Reg II		Reg II		
III	HIP/PAP Reg III? Reg IIId	Reg III/PAP II PAP I PAP III	Reg IIIa Reg IIIß/PAP Reg III? Reg IIId	INGAP	PTP
IV	Reg IV		Reg IV		

HIP, hepatocellular carcinoma, intestine and pancreas; **PAP**, pancreatitis-asssociated protein; **PTP**, pancreatic thread protein; **INGAP**, islet neogenesis-associated protein.

Figure 22. REG gene family in human, rat, mouse, hamster and cow

5.4 REG GENES AND REGENERATION

In the mouse, *reg* genes have a common gene structure with 6 exons and 5 introns and encode homologous 165-175 aa proteins. Both *reg*1 and *reg*2 mRNAs are detected in the exocrine pancreas and hyperplastic islets of aurothioglucose-treated mice, but not in the normal islets. *Reg* 3alpha, *reg* 3beta and *reg* 3gamma are expressed weakly in pancreas, strongly in the intestinal tract, but not in hyperplastic islets. *Reg* 3delta displays a widespread occurrence: exocrine pancreas and hyperplastic islets (together with *reg* 1 and *reg* 2) and intestine or colon (together with the other *reg* 3 subtypes).

Mouse *reg* 3beta was shown to act as a Schwann cell mitogen associated with the regeneration of motor neurons. From these reports, it is reasonable to assume that reg proteins, especially type III reg proteins, act as growth factors in alimentary tract, liver, and pancreatic acinar cells and neuronal cells, as type I reg protein acts on pancreatic beta cells. Mouse *reg* 4 was assigned to a different chromosome, like REG 4 in humans, chromosome 3.

Although much is known on the genetic, transcriptional regulation and tissue expression of REG genes, very little information is currently available on the receptor(s) utilization and intracellular signalling activated by REG genes in the target cells. A receptor for reg protein that mediates the growth signal of Reg proteins for beta cell regeneration has been identified[195]. The expression of the REG receptor, however, is not increased in regenerating islets as compared with that in normal islets; this

observation suggests that the regeneration of pancreatic beta cells is primarily regulated by the increased expression of REG genes[196].

In summary, the main functional role of REG genes in different tissues appears to be the promotion of tissue homeostasis and regeneration upon tissue injury by a wide range of insults. In this regard, beta cells have been shown to be susceptible to damage from numerous agents such as immunological abnormalities, virus infections, irradiation, and chemical substances, leading to local inflammation in and/or around pancreatic islets. In particular, the close relationship between beta cells damage, resulting inflammation and consequent up-regulation of REG genes in the pancreas has been recently dissected, with a prominent role for in situ IL-6 production during the inflammatory process. It was found that IL-6 plays a role in the activation of REG genes and that PARP inhibitors such as nicotinamide and 3-aminobenzamide enhanced the induction.

IL-6 stimulation induces the formation of an active transcriptional complex for REG, in which PARP is involved.

PARP was shown to bind the IL-6-responsive element of REG gene, forming the active transcriptional DNA/protein complex for REG gene expression.

The formation of the active transcriptional complex was further enhanced by the inhibition of the autopoly(ADP-ribosyl)ation of PARP. When the PARP is not poly(ADP-ribosyl)ated in the presence of PARP inhibitors, the transcriptional complex is stabilized and the RG gene transcription is maintained[197,198]. Reg protein then produced in beta cells acts as an autocrine/paracrine growth factor on beta cells via the REG receptor.

DNA replication in beta cells occurs and the beta cell regeneration is achieved.

In addition to regenerating islets in 90% depancreatized rats receiving PARP inhibitors[199], reg gene expression was also observed in the phase of transient beta cell proliferation such as in pancreatic islets of BB rats during the remission phase of diabetes[200], in islets of NOD mice during active diabetogenesis[201] and pancreatic ductal cells, which are thought to be progenitor cells of beta cells, during differentiation and proliferation in a mouse model of autoimmune diabetes[202] and inflammation in and/or around islets was involved in these cases.

Despite up-regulation of REG genes in the pancreas is a prominent feature in the course of the autoimmune insulitis, it is clear that regeneration of the beta cells induced by REG genes is not capable of preserving beta cell function, as patients with T1D and animal models progress towards beta cell destruction and overt diabetes. Thus, it has been suggested that additional mechanisms are implicated in impairing the attempt of REG genes to restore the beta cell mass upon inflammation-mediated injury. Recent evidence suggest that an autoimmune process directed against the REG genes themselves might be responsible for the impaired function of REG genes. In this regard, it has been clearly shown that REG might act as novel autoantigens in T1D and thus become a target of the autoimmune process, possibly further promoting chronic inflammation and contributing to the disease pathogenesis[203].

On islets from a patient who died at the onset of T1D, cDNA encoding HIP/PAP was identified. Data in literature demonstrate that NOD mice display spontaneous T-cell responses to HIP/PAP. These T-cells can home to the pancreatic islets and can transfer disease when coinjected with CD8+ T-cells from diabetic NOD mice. It is possible that these T-cells require the cytokine/chemokine signals produced by islet-infiltrating CD8+ cells in order to leave the pancreatic lymph nodes and migrate to and remain in the islets. Alternatively, islet-infiltrating CD8+ cells might cause upregulation of production/secretion of REG from the islets, which then activates T-cells resting in the pancreatic lymph nodes, causing them to accumulate in the islets.

It has also been demonstrated that IL-6 mediates upregulation of REG production/secretion from isolated human islets. If such a mechanism indeed plays a role in vivo, we could imagine a scenario where during the progressive islet inflammatory process, increased amounts of IL-6 are released either from the infiltrating cells or (when triggered by their cytokines, such as ?-interferon) from the islets themselves[204,205]. This would then lead to upregulation of REG expression; potentially, proliferation of T-cells recognizing the autoantigen HIP/PAP; and perhaps a progressive acceleration of the disease process. The fact that islets respond with upregulation of REG expression/secretion when injured by inflammation would fit the role the REG family has been reported to play. Its function in response to islet inflammation might be to support islet regeneration and to protect the islet from inflammatory damage. If this were the case, overexpression of a putative islet regeneration protein that

has the potential to act as an autoantigen (capable of generating an autoimmune response against it) could create a vicious cycle, accelerating the immune process leading to diabetes. Consistent with this hypothesis, the presence of autoantibodies against REG has been demonstrated in diabetic patients. In a recent study carried on in Japan, 24.9% T1D and 14.9% T2D patients tested positive for anti-Reg 1alpha antibodies. Although confirmatory studies are needed, evidence accumulated suggests that a continuous process of destruction/regeneration take place in the pancreas during autoimmune insulitis and that Reg genes play a fundamental role not only in the attempt to regenerate the reducing beta cell mass, but also as a target of the autoimmune process itself.

5.5 AIM OF THE STUDY

The aim of this project is to evaluate the role of REG genes and proteins in beta cell regeneration in chronic inflammatory autoimmune diseases. In order to do so, the first part of the study will provide strong observational evidence of the involvement of REG genes and proteins in humans. Patients affected by T1D, T2D, Sjogren's Syndrome (SS), Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA) will be studied from several points of view and all the data will be correlated, compared with healthy controls and statistically analyzed. Diabetic patients will be grouped as follow: T1D newly diagnosed, T1D with long lasting disease and T2D. Several parameters will be considered: age, sex, disease duration, BMI, c-peptide, insulin levels, HbA1c, therapy, presence of diseases related auto-antibodies, other autoimmune disorders. Patients genetic profile will

be also clearly analyzed. Genes polymorphisms such as HLA, INS, PTPN22, CTLA4, will be evaluated. Once patients will have been studied from all these points of view and a clear picture of their genetic, metabolic and immunologic state will have been drawn, REG data will be considered and correlated. First all human Reg proteins level will be measured in sera: Reg 1alpha, Reg 1beta, Reg2, HIP/PAP, PAP IB, Reg 4. Than we will evaluate the presence of auto-antibodies against all Reg proteins from a qualitative and quantitative point of view. As IL-6 inflammation has been shown to be critical for the induction of REG genes, in this work we will also measure the cytokine levels in the sera of all subjects. The dosages of other inflammatory cytokines levels will be evaluated to be able to define clearly the role of inflammation on REG genes induction. Cytokines that will be considered are INF-?, TNFa, IL-4, IL-18.

From the genetic point of view patients genome will be studied to evaluate the presence of REG genes polymorphisms. This analysis will be carried out considering the genetic differences within the disease: only subject affected by the autoimmune disorder will be included but they will be grouped according to the different degree of cell regeneration. The degree of regeneration will be assessed by the blood levels of Reg proteins. This first part of the study will allow us to delineate the involvement of REG genes and proteins in a clear and complete way. These data will also define the value of serum Reg proteins levels and Reg auto-antibodies as bio-marker of regeneration and disease respectively.

NOD mice is an animal model of autoimmune diseases as these mice spontaneously develop the diseases. This is why we believe that they are the most appropriate animal for our functional studies.

The first part of the project in mice will delineate the same complete observational picture as in the human study. In order to do so we will focus on the assessment of murine REG genes expression in the target organ, pancreas. In a large population of NOD mice RNA will be extracted from tissues and reverse transcribed into cDNA. The mice should be culled at different life and disease stages allowing to delineate a time course expression of REG genes. The genetic analysis will involve all murine *reg* genes: *reg1, reg2, reg3alpha, reg3beta, reg3gamma, reg3delta, reg4*. One of this study's aim is to correlate reg genes expression and disease intensity evaluated trough the histological level and quality of the inflammatory infiltrates. NOD mice pancreas will be graded histologically from a quantitative and qualitative point of view. To complete the evaluation of reg involvement in NOD mice, we will measure animal sera reg proteins levels and estimate the presence of anti reg specific auto-antibodies.

To be able to analyze statistically these data, all above points will be studied in control mice (balb/c) sex/age matched.

Once observational data are achieved also in NOD mice, functional studies will be carried out in order to understand the patho-physiological mechanisms involved in cell regeneration. First the study will identify which cells express Reg proteins and which specific reg protein is associated with different cell types. Immunohistochemistry staining will localize reg proteins and other cells in pancreas using specific anti Reg

antibodies and cell markers. As IL-6 inflammation has been shown to be critical for the induction of *reg* genes, in this work it will be investigated the gene and protein expression when IL-6 is added to cell culture.

All these experiments were conceived in order to state the role of *reg* genes and proteins in autoimmune disorders and prove if and how our hypothesis may be validated.

According to the data available to date we believe that the following hypothesis on Reg role in autoimmune disorders should be considered.

In autoimmune diseases, target cells are destroyed by self-reacting cells. In T1D auto-reactive inflammatory cells recognize antigens on beta cells and destroy the islets trough T- and B- cells driven mechanisms. The autoimmune process, with cell rupture and antigen exposure, enhances inflammation within the islets determining the development of insulitis. More inflammatory cells are gathered and cytokines and chemokine take part in the destructive process. This damaging could than stimulate the cells to replicate in order to replace the original cell mass. It is known that adult precursors cells can differentiate in beta cells in injured adult mouse pancreas. These progenitors have been located in the ductal lining. The possible regenerating process, together with inflammatory stimuli, would then lead to up-regulation of Reg genes expression and consequent Reg protein secretion. The fact that tissues respond with up-regulation of Reg expression/secretion when injured by inflammation would fit the role the

Reg family of proteins has been reported to play. The over expression of regenerating proteins would determine the presence of new potential antigens. These Reg proteins would be capable of generating an autoimmune response against themselves. T-cells in fact could recognize the antigens, B-cells would be activated and would produce antibodies against them. This process would lead to a progressive acceleration of the disease and even create a vicious cycle: genetically susceptible subjects develop autoimmunity towards pancreatic islets; beta cells are destroyed and overt disease manifests; precursors cells try to replace the destroyed cells; precursors themselves, beta cells or even environmental cells express Reg genes in order to stimulate the regenerating mechanism; beta cells may in fact secrete Reg proteins that act on precursors; Reg proteins may be secreted by precursors and act directly on these cells; the inflammatory process may play a stimulating role in enhancing Reg genes expression trough cytokines and chemokines; Reg proteins are secreted in large quantity and stimulate the immune system against themselves; auto-antibodies are produced against Reg proteins; the cells that express and secrete Reg proteins are destroyed and their attempt to regenerate the beta cell mass fails. We believe that this vicious cycle occurs in T1D and that Reg genes and proteins have a fundamental role in maintaining it. Our experiment will prove if this innovative hypothesis is true and clearly define the mechanisms involved in this regenerating-destructing cycle.

From the inflammation point of view we will define the role of several molecules and clarify whether they are involved in the induction of Reg genes. Cytokines and chemokines serum levels will be evaluated, their

effect in cultures and the presence of promoters on Reg genes. We also believe that the same vicious cycle that may be involved in T1D pathophysiology, could be active in the other autoimmune diseases. Data obtained from this work will clarify the possible involvement of Reg genes and protein also in SS, RA and SLE.

5.5.1 SPECIFIC AIMS IN HUMAN SAMPLES

1. To determine Reg proteins levels in sera of several groups of diabetic patients at different stages of life/disease and with different metabolic conditions, SS patients, RA patients and SLE patients.
2. To evaluate the presence of auto-antibodies against all Reg proteins from a qualitative and quantitative point of view in all above subjects.
3. To correlate the Reg proteins blood dosages with several parameters: disease duration, age, c-peptide, insulin levels, HbA1c, T1D related genes polymorphisms (HLA, INS, PTPN22, CTLA4), IL-6 blood levels, other inflammatory cytokines levels in blood, auto-antibodies production.
4. To evaluate possible Reg genes polymorphisms.
5. All above points in healthy controls sex/age matched.

5.5.2 SPECIFIC AIMS IN MURINE SAMPLES

1. To evaluate the expression of all *reg* genes in pancreas of NOD mice at different stages of life/disease.
2. To localize *reg* genes and proteins within the target organ; identify the cells that express the genes and proteins.
3. To determine reg proteins levels in sera of NOD mice.

4. To evaluate possible *reg* genes polymorphisms.

5 All above points in control mice (balb/c) sex/age matched.

5.6 WORK CARRIED OUT TO DATE IN HUMAN AND IN MICE

The work carried on to date involves a) a human component and b) an experimental animal model: the NOD mouse.

a) In humans.

1. Serum levels of Reg1alpha protein in the following patient groups were measured: T1D subjects (newly diagnosed and long standing), T2D subjects, SS patients, RA patients, SLE patients, Controls sex/age matched (normal subjects).

2. The correlation between C-peptide (marker of beta cell function), HbA1c, duration of disease and Reg1alpha (marker of beta cell regeneration) protein levels was evaluated.

3. The genetic associations between Reg1alpha levels and autoimmune diseases related genes polymorphisms (HLA, INS, PTPN22, CTLA4) was evaluated.

4. Finally autoantibodies against Reg 1alpha in T1D subjects and controls were measured.

b) In mice.

1. *Reg* genes (*reg1, reg2, reg3alpha, reg3beta, reg3gamma, reg3delta, reg4*) expression in the pancreas of pre-diabetic and diabetic mice.

2. The histological degree of inflammation and infiltration in target organ.

3. All above points in control mice (C57BL/6 and BALB/C mice).

5.7 HUMAN STUDIES

5.7.1 MATERIALS AND METHODS

Subjects enrolled in this project have been identified in previous studies through the IMDIAB network and genetic/immunological data are already available (n= 1000) (see publications IMDIAB group). These patients come from the IMDIAB group which was formed in 1988 and has some of the largest worldwide experience in identifying and studying subjects with recent onset T1D. Patients that will be part of the study are all Caucasian and come from the same geographical area, ensuring the important feature to be a homogeneous cohort. In fact, the vast majority of these subjects live and are diagnosed in the Lazio region (central Italy) and once diagnosed are enrolled in follow- up trials where they are monitored by the same team of physicians and paramedical staff for at least two years after diagnosis. The age distribution of the patients referred to the IMDIAB clinics, similar between females and males, is as follows: 4,3% between 5 and 7 years old, 16.2% in the 7-10 range, 40% between 10-17, 7.2% and 32.3% in the 17-20 and 20-45 ranges, respectively. The mode peak time of onset of T1D is 9 years for males and 12 for females. These patients have a baseline C- peptide of 0.26±0.22 nmol/L (0.00-1.75) while the glucagon stimulated C- peptide is 0.47±0.38 (0.02-2.51).

The prevalence of GAD and IA-2ic antibodies in our population is 65% and in 59% respectively, while both antibodies are present in the 40% of the patients. BMI at diagnosis of these patients was 18.9±3.8 (range

11.1-35.6) and they are on a mean insulin dose of 0.62±0.35 UI/kg bw. The low BMI and the overall clinical features at clinical diagnosis, their well already proved suitability in successful clinical studies make these patients an ideal group to compare with DD subjects. Blood samples were collected and stored for analysis.

Serum levels of Reg1alpha protein in the following patient groups were measured:

- T1D subjects (31 newly diagnosed and 56 long standing),
- 63 T2D subjects,
- 64 controls sex/age matched (normal subjects).

The protein was measured using the BioVendor Human Reg 1alpha ELISA kit, which is a double polyclonal sandwich enzyme immunoassay for quantitative measurement of human Reg 1alpha protein in serum and plasma.

5.7.1.1 ELISA

The Human Reg 1alpha ELISA is a double polyclonal sandwich enzyme immunoassay for quantitative measurement of human Reg 1a protein in serum and plasma.

In the BioVendor Human Reg 1alpha ELISA, calibrators, quality controls and samples are incubated with polyclonal anti-human Reg 1alpha antibody coated in microtitration wells.

After one-hour incubation and washing, polyclonal anti-human Reg 1alpha antibody labelled with horseradish peroxidase (HRP) is added to the wells

and incubated with captured Reg 1alpha. After one-hour incubation and another washing step, the remaining conjugate is allowed to react with tetramethylbenzidine susbstrate and H_2O_2. Adding acidic solution will stop the reaction, and absorbance of the resulting yellow colour product can be measured spectrophotometrically at 450nm. The absorbance is proportional to the Reg1a concentration. A standard curve is obtained by plotting absorbance values versus Reg 1alpha concentrations of calibrators; this standard curve is used to determine concentrations of unknown samples.

The calibration curve is constructed by plotting the absorbance (Y) of standards versus *log* of the known concentration (X) of standards, using the four-parameter function. The results are reported as the Reg 1alpha (ng/ml) concentration in samples.

The actual amount of Reg 1alpha in the original blood sample has been assessed by multiplying the assay result by dilution factor 50 (e.g. 13,5 ng/ml x 50 gives 675 ng/ml).

5.7.1.2 WESTERN BLOT

Western blotting was used to estimate the presence of autoantibodies anti Reg 1alpha protein in sera. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) involves separation of proteins, the blotting of these proteins onto a nitrocellulose membrane and their detection using specific antibodies. The polyacrylamide gels are run in the presence of the anionic detergent SDS that denatures and binds to proteins in a constant-weight ratio leading to identical charge densities for the

denatured proteins. Thus, the SDS-protein complexes migrate in the polyacrylamide gel according to size and not charge.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Reg 1alpha proteins migrated in response to an electrical field through the pores of a SDS polyacrylamide gel matrix toward the anode, under denaturing conditions. A discontinuous buffer system was used, with buffers of different pH and composition to generate a discontinuous pH and voltage gradient in the gel.

Polyacrylamide gels form after polymerisation of monomeric acrylamide into polymeric polyacrylamide chains and cross-linking of the chains by N, N'-methylenebisacrilamide. The discontinuous polyacrylamide gel consisted of an upper 4% stacking gel with large pore size where proteins were concentrated and a 10% lower separating gel with smaller pore size where they were separated.

The gel, Invitrogen NuPAGE 4-12% Bis-Tris Gel, 1.5mm x 15 wells, was assembled in the electrophoresis tank according to the manufacturer's instructions (Bio-Rad) and electrophoresis running buffer poured into both the inner and outer chambers.

The running buffer (pH 7.7) composition is 50mM MOPS, 50 mM Tris Base, 0.1% SDS, 1mM EDTA. Equal amounts of protein samples (~5µg) were denatured by five minutes boiling at 95°C in LDS buffer, H_2O and the reducing agent β-mercaptoethanol in the following quantities: LDS buffer 2.5µl, reducing agent 1µl, 10µl Reg 1alpha protein and H_2O in different quantity to a maximum of 15µl total.

The protein samples were loaded into the gel lanes and then separated by electrophoresis at 120V for 90 minutes.

A pre-stained molecular weight marker containing labelled proteins of specific molecular weights was run in parallel and used to visualise protein migration and calculate protein molecular weight.

Blotting onto nitrocellulose membrane

Following electrophoresis, proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane, with 0.45µm pore size, using a mini transblot apparatus (Bio-Rad). Membrane, gel, two pieces of filter paper and two fibre pads per gel were soaked in the transfer buffer (pH 7.2), containing 25 mM Bicine, 25 mM Bis-Tris and 1 mM EDTA, to equilibrate for 10 minutes.

A transfer cassette was assembled consisting of a sandwich containing layers of fiber pad, filter paper, gel, membrane, filter paper and fiber pad. The cassette was then placed in the blotting tank according to the manufacturer's instructions, all fully covered with transfer buffer and with an ice block to prevent excessive heating of the buffer and consequent protein denaturation.

Electrophoretic transfer of negatively charged proteins from the gel to the membrane was achieved by the application of a 100V current for 60 minutes.

Equal protein loading and transfer efficiency to the membrane was verified using the protein dye Ponceau S. The membrane was rocked gently with 0.1% Ponceau S staining solution for 10 minutes and then destained with

water until pink protein bands appeared on the membrane. To completely remove the staining the membrane was than washed 3 times with PBS+0,1% Tween solution.

Immunodetection of proteins

Prior to immunodetection the membrane was blocked in a solution of Blocking buffer containing PBS, 2%Skim Milk, 0.1% Tween 20, for 1hr at room temperature on a rocker platform to minimize the non specific binding of the antibody.

The membrane was then incubated with patients sera, eventually containing primary antibodies against Reg1alpha, diluted in Blocking buffer on a rocker platform for 2hrs.

Following three 5 minutes washes of the membrane in PBS+0.1% Tween 20, to remove unbound antibody, the membrane was incubated at room temperature for 1hr on a rocker platform with goat anti-human-IgG secondary antibody diluted in Blocking buffer. The membrane was again washed, as above, to remove unbound secondary antibody. Finally three 5 minutes washes of the membrane in PBS+0.1% Tween 20, to remove unbound antibody. The membranes were than incubated with Super Signal West Dura Extended Duration Substrate Antibodies (Pierce) for 5 minutes and than developed.

Enhanced Chemiluminescent (ECL) detection

Immunocomplexed bands were detected by enhanced chemiluminescence.

The membrane was exposed, under red light, to light-sensitive autoradiography film (Hyperfilm ECL) for various time periods. The film was than washed in the Developer and Fixer buffers. If antibodies are present in the sera a black band shows on the film.

5.7.1.3 C-PEPTIDE AND HbA1c MEASUREMENT

The correlation between C-peptide (marker of beta cell function) and Reg1alpha (marker of beta cell regeneration) was studied.

Baseline C-peptide was measured fasting in the morning using a radioimmunoassay method using a commercially available kit (Bio-Rad Laboratories). The reference range of fasting C-peptide stabilised in 64 control subjects (matched for age and sex, with no family history of T1D) was > 0.4 nmol/l with intracoefficient and intercoefficient variability between 10 and 15%, respectively.

Our laboratory has participated in the world-wide standardization of C-peptide assay and contributed to the definition of the standard[206]. The comparison between laboratories in single specimens, expressed as %CV, ranged from 12.8 to 33.4% (our laboratory 16%).

We investigated the correlation between HbA1c, duration of disease and Reg1a (marker of beta cell regeneration). Glycated haemoglobin (normal range 4.0–7.0%) was measured centrally (Bio-Rad Laboratories, Milan, Italy).

5.7.2 RESULTS

1. Reg1alpha protein was highly detected in the sera of diabetic patients.

The results showed a significant difference between newly diagnosed T1D patients and controls (p=0.002), long standing T1D patients and controls (p=0.001) but no difference was found between newly diagnosed and long standing T1D patients (p=0.22). A significant difference was also found between newly diagnosed T1D and T2D patients (p=0.006) and T2D patients and controls (p<0.001) but no difference was present between long standing T1D and T2D patients (p=0.09).

The correlation between C- peptide (marker of beta cell function), HbA1c, duration of disease and Reg1alpha ("possible" marker of beta cell regeneration) protein levels was evaluated.

No correlation was found between Reg 1alpha serum levels and C- peptide values; no correlation was found between Reg 1alpha serum levels and HbA1c values; no correlation was found between Reg 1alpha serum levels and age nor disease duration.

The genetic associations between Reg1alpha levels and T1D related genes polymorphisms (HLA, INS, PTPN22, CTLA4) was evaluated but no association was found.

These results are the evidence of the presence and involvement of Reg 1alpha protein in T1D and pancreas damage. The protein secretion was detected both at the beginning and the long course of the disease. This observation suggests the fact that the pancreas attempts to regenerate the beta cell mass at all times and supports our hypothesis of a vicious cycle that perpetuates during all the disease course. In particular, the high levels of the protein in T2D, suggest that Reg secreting cells are actually destroyed by an autoimmune process against Reg proteins only in subjects

with a genetic susceptibility to autoimmunity. In T2D pancreas in fact is not destroyed by autoimmunity or inflammation, its regenerative environment is only due to its attempt to produce more insulin. In this type of diabetes the low sensitivity to insulin in the peripheral organs determines an increase in the need of the hormone and a subsequent increased production. This would stimulate beta cells precursors to regenerate and induce REG genes expression. On the other hand, in autoimmune diabetes, the hypothesis of this project states that Reg secreting cells become the target of an autoimmune process against Reg proteins and are destroyed while attempting to restore the beta cell mass.

The absence of correlation between C- peptide, HbA1c, duration of disease and Reg1alpha protein levels is the confirmation of the fact that the metabolic condition is not involved in Reg genes induction. Insulin and high glucose levels do not inhibit the secretion of Reg 1alpha protein. In fact, if the hormone or the sugar had an inhibitory effect on Reg genes, we wouldn't have found high levels of the protein in T2D. The lack of correlation with the genetic polymorphisms associated with T1D is due to the fact that Reg genes expression, according to our hypothesis, is mainly a secondary phenomenon. Reg genes are expressed subsequently to cell loss and protein secretion is a consequence of the disease not a pathogenic factor.

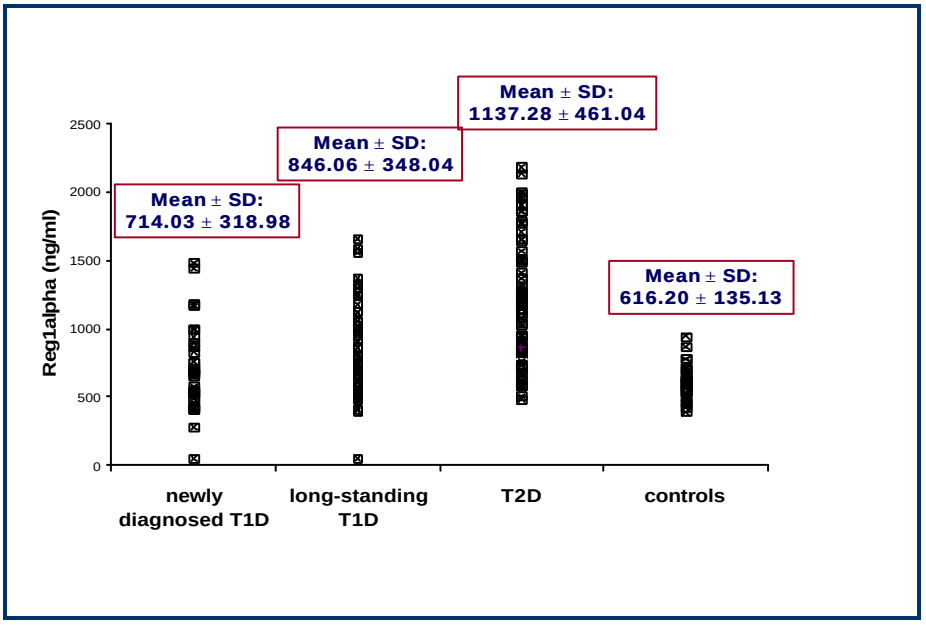


Figure 23. Reg 1alpha serum levels in T1D, T2D and control subjects.

2. An interesting result was shown by Reg1alpha serum levels in the other autoimmune diseases. In fact significant difference was found between SS patients and controls (p=0.0001), RA patients and controls (p=0.0001), but no difference between SLE patients and controls.

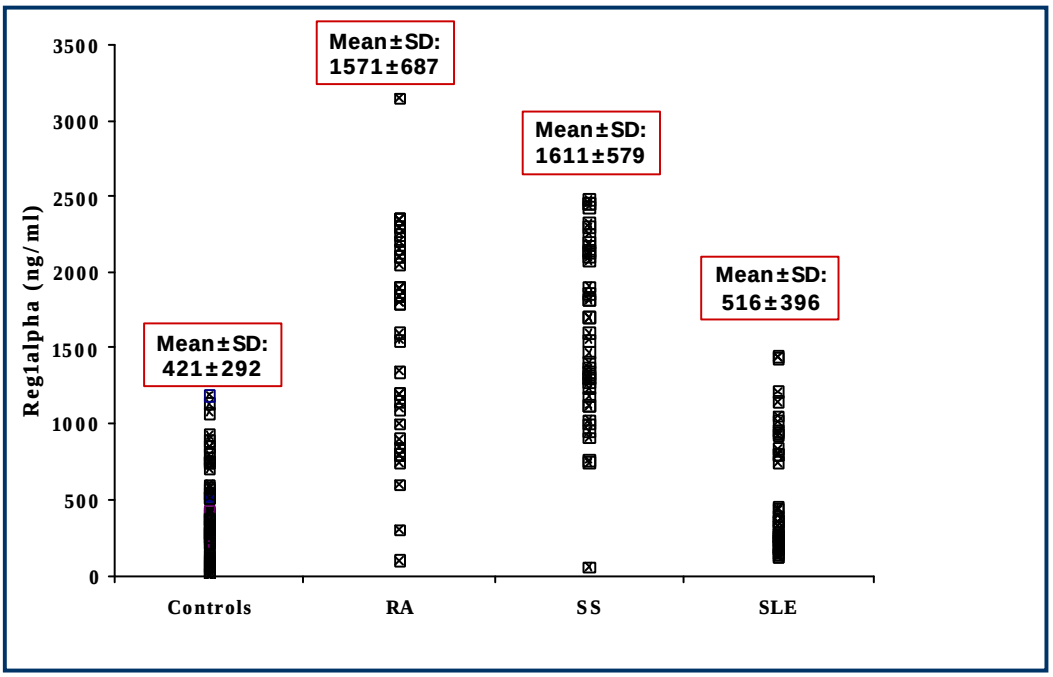


Figure 24. Reg 1 alpha serum levels in autoimmune diseases.

3. the genetic associations between Reg1alpha levels and T1D related genes polymorphisms (HLA, INS, PTPN22, CTLA4) was evaluated but no association was found.

4. Finally autoantibodies against Reg 1alpha in T1D subjects and controls were measured. Autoantibodies were present in T1D newly diagnosed patients but we have still not conduced the experiment in control subjects.

Chiara Guglielmi

The Endocrine Society
8401 Connecticut Avenue, Suite 900,
Chevy Chase, MD 20815
301-941-0200
www.endo-society.org

March 8, 2007

Dear Dr. Guglielmi:

Re: Reg1-[alpha] as a Marker of beta Cell Regeneration in Type 1 Diabetes.

The abstract listed above has been accepted for an **Oral Presentation** at ENDO 2007, The Endocrine Society's 89th Annual Meeting, June 2 – 5 in Toronto, Canada. This year for your convenience, we have added a disposition web site. The site allows you to view and print the official abstract notification letter and access guidelines for preparation of your presentation.

On behalf of the Annual Meeting Steering Committee, we look forward to welcoming you to the meeting and hope that your colleagues and coauthors will also have the opportunity to participate in this important event. See you in Toronto!

Sincerely,

William F. Young, Jr., MD
Chair, Annual Meeting Steering Committee

Andrea C. Gore, PhD
Basic Chair, Annual Meeting Steering Committee

Peter J. Trainer, BSc, MD
Clinical Chair, Annual Meeting Steering Committee

5.8 MOUSE SAMPLES

5.8.1 MATERIALS AND METHODS

The mice used in this study come from the Ba/NOD colony, established in 1987 at St. Bartholomew's Medical College, London, and have been employed to:

1. Create a large biobank of pancreas stored in RNAlater and OCT at - 80° C, and sera; samples were collected at different stages of life/disease.

2. Investigate murine *reg* genes (*reg1, reg2, reg3alpha, reg3beta, reg3gamma, reg3delta, reg4*) expression in the pancreas of pre-diabetic and diabetic mice.

3. Investigate the histological level and quality of inflammation and infiltrate in the target organ.

4. All above points in control mice (BALB/C mice).

106 female NOD mice were sacrificed at diverse age and different stages of life disease: 4, 8, 12, 16, 20, 24, 28, 32 over 32 weeks. Pancreas was extrated and used for histology and RT-PCR analysis.

In all female NOD mice murine *reg* genes expression was evaluated in the pancreas. Mice pancreas showed highly expression of all *reg* genes and the gene expression peaked at onset of disease (between 12 and 15 weeks). Only *reg* 4 was not expressed.

Both mouse Beta Actin and 18S were used as endogenous controls for the analysis of the RT-PCRs. RNA was extracted from samples using the Quiagen RNeasy mini kit and than reverse-transcribed into complementary DNA (cDNA) using a reverse transcriptase before being

amplified. mRNA was reverse transcribed to cDNA using the Thermoscript RT-PCR System for First-Strand cDNA Synthesis (Invitrogen). The real-time PCR were than run in duplicate on 384-well PCR plates with an equal loading of 10ng of cDNA/well and detected using the ABI PRISM 7900HT Instrument (Applied Biosystem). The OCT embedded samples were used for the Haematoxylin and Eosin staining to evaluate the infiltrate organization and its relationship with the surrounding tissue. Four microns sequential cryostat sections were obtained by cutting the specimens in a cryostat. The histology samples were classified and graded considering the infiltrates quality, peri- islet or intra- islet inflammatory infiltrates, and quantity, using a 3 degrees scale according to the number of inflammatory cells visualized. The correlation between gene expression and the histological data showed the same peak at disease onset: both Reg genes expression and islet inflammatory infiltrate have the maximum degree around the 12th – 15th week.

The same analyses were carried on 20 BALB/c mice. Also these animals were sacrificed at diverse age (4, 8, 12, 16, 22) and samples from pancreas were extrated and used for hystology and RT-PCR analysis. All *reg* genes showed the same pattern: gene expression decreases drammatically from 4 weeks to 22 weeks.

This data was never shown before in literature and is a very important observation as it hepls clarifying *reg* genes function in the pancreas of both NOD and healthy mice. We believe in fact that during the normal growth

of the pancreas in early life, *Reg* genes have an active role in inducing the phisiological expanding process. Once the organ is formed, *reg* genes gradually decrease their degree of expression. In the NOD mice instead, Reg genes are re activated later in the animal life (12-15 weeks) consequentially to the organ damage. In fact, autoimmunity destroys beta cells and the loss activates *Reg* genes at disease onset exactly as our RT-PCR data showed. The gene expression in NOD pancreas after 18 weeks decreases as an autoimmune process probably starts against *Reg* secreting cells. BALB/C pancreas instead stop expressing *Reg* genes once the organ has reached its adult volume at 15 weeks.

5.8.1.1 EXTRACTION OF TOTAL RNA FROM MOUSE TISSUES.

RNA was extracted using the Quiagen RNeasy mini kit according to manufacturer' instructions. Tissues stored at -80°C in RNA Later were defrost in ice, weighted and then cut in order to obtain approximately 20mg (30mg of tissue is the maximum amount suggested in the protocol to avoid reduction in RNA yield and purity). Tissues were placed in a sterile, RNAse free tube and 600 µl of buffer RLT (containing denaturing guanidine thiocyanate and with the addition of 10µl of fresh beta-Mercaptoethanol/ml of RLT buffer) were added. Tissues were homogenised using a rotor–stator homogenizer until the sample was uniformly homogeneous (usually 1 min). Tissue lysates were centrifuged for 3 minutes at maximum speed (13000rpm) in a microcentrifuge and supernatant was transferred to a new RNAse free microcentrifuge tube. An equal volume of 70% ethanol was added to the lysate and immediately

mixed by pipetting in order to precipitate RNA, which remains in the aqueous phase. An aliquot (700µl) of the sample was added to the RNeasy mini column placed in a 2 ml collection tube and centrifuged for 15 s at =8000 x g (=10,000 rpm) to allow the RNA to bind to the silica column. The flow-through was discarded and the remaining sample (approximately 500µl) added to the RNeasy mini column and centrifuged as above.

To avoid any possible DNA contamination, a DNase step was included according to manufacturer' instruction. 350 µl of Buffer RW1 were added to the column, centrifuged for 15 s at =8000 x g (=10,000 rpm) and the flow-through discarded. 10 µl of DNAse I stock solution (previously prepared by dissolving solid DNase I (1500 Kunitz units) in 550 µl of RNase-free water) were added to 70µl of Buffer RDD, gently mixed and added to the RNeasy mini column silica-gel membrane. Following 15 min incubation at room temperature (RT), 350 µl of Buffer RW1 were added to the column, centrifuged for 15 s at =8000 x g (=10,000 rpm) and the flow-through discarded.

The RNeasy column was transferred into a new 2 ml RNase free collection tube. In order to wash away contaminants (residual DNA and proteins) in the organic phase, 500 µl of Buffer RPE were pipetted onto the RNeasy column and centrifuged for 15 s at =8000 x g (=10,000 rpm) to wash the column. The flow-through was then discarded and another 500 µl Buffer RPE added to the RNeasy column. The tube was centrifuged for 2 min at =8000 x g (=10,000 rpm) to dry the RNeasy silica-gel membrane and the flow-through discarded. To eliminate any chance of possible Buffer RPE

carryover the tube was centrifuged again for 1 min at full speed (13,000 rpm).

For elution, the RNeasy column was transferred to a new RNAse free 1.5 ml collection tube, and 30 µl of RNase-free water were pipetted directly onto the RNeasy silica-gel membrane and the tube centrifuged for 1 min at =8000 x g (=10,000 rpm). The elution was immediately frozen at -80°C until required.

5.8.1.2 REVERSE TRANSCRIPTION PCR

In Reverse-Transcription PCR (RT-PCR), mRNA is converted to complementary DNA (cDNA) using a reverse transcriptase before being amplified. RNA was reverse transcribed to cDNA using the Thermoscript RT-PCR System for First-Strand cDNA Synthesis (Invitrogen). Briefly, RNA (quantity depending on concentration) from each sample was mixed with 1 µl of Oligo(dT)20 Primers (50 µM) and 2 µl of 10 mM dNTP mix and brought to a 12 µl volume reaction with DEPC-treated water in a 0.5ml PCR tube. After brief spinning down in a microcentrifuge, samples were incubated for 5 min at 65°C to denature RNA tertiary structure and samples were immediately cooled on ice to allow RNA and oligos to anneal. For the final reaction, 8 µl of a master mix containing 1 µl of ThermoScript™ RT (15 U/µl), 1 µl of 0.1 M DTT, 1 µl of RNaseOUT™ Ribonuclease Inhibitor (40 units/µl), 1 µl of DEPC-treated water and 4 µl of 5X cDNA synthesis buffer were added. After mixing and a brief spin down of the tubes, the reverse transcription to cDNA was run in a PCR machine (Applied Biosystems 9700) for 1h at 50°C and the reverse

transcriptase was inactivated at 85°C for 5 min. In order to remove the original RNA that could interfere with the quantitative real-time PCR analysis, RNA digestion was performed using 1µl of E.Coli RNase H (2 units/µl) at 37°C for 20 min. Finally, the completed cDNA strand was diluted to a final concentration of 10ng/µl with DEPC-treated water and stored until used.

5.8.1.3 QUANTITATIVE TAQMAN REAL-TIME PCR.

PCR involves amplification of a specific DNA sequence, which spans between two sequences of primers. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each cycle (in real time) as opposed to the endpoint detection by conventional quantitative PCR methods; it is based on the detection and quantitation of a fluorescent reporter whose signal increases in direct proportion to the amount of PCR product in the reaction.

For quantitative TaqMan real-time evaluation of mRNA expression levels of mouse genes, sequence-specific primers and probes from Applied Biosystems were used. Each gene expression assay contains, together with the forward and reverse primers, a TaqMan MGB probe with a FAM reporter dye at the 5' end. Within the probe, the dye is linked to a non-fluorescent quencher; during the polymerase reaction, the probe is detached from the cDNA and the quencher is released allowing fluorescence emission from the reporter dye.

The real-time PCR were run in duplicate on 384-well PCR plates (Applied Biosystems) with an equal loading of 10ng of cDNA/well and detected

using the ABI PRISM 7900HT Instrument. The thermal cycling conditions used comprised a 2 min UNG activation step at 50°C, a 95°C Taq polimerase enzyme activation step for 10 min, and cycles of 95°C denaturation for 15 sec and 60°C anneal/extension for 60 sec. Results were then analysed after 45 cycles of amplification using the ABI PRISM 7900HT Sequence Detection System Version 2.1 (SDS 2.1). Relative quantification was measured using the Comparative Ct (Threshold Cycle) Method. Two different endogenous controls (human or mouse beta-actin and mammalian 18S) were used to normalize for the cDNA of each sample. The ?Ct for each of the duplicate (Ct of the target gene minus Ct of the endogenous control) and then the average ?Ct of the duplicates were calculated. When a single value within each duplicate differed substantially from the other value the skewed value was excluded from the analysis. To calculate the ??Ct, the ?Ct of each sample was subtracted to the chosen reference sample. The relative quantity was then calculated following the equation RQ= $2^{-??CT}$ where 2 represents doubling of the amount of the product of amplification after each PCR cycle.

5.8.2 RESULTS

In 106 NOD mice we evaluated *reg* genes (*reg1, reg2, reg3alpha, reg3beta, reg3gamma, reg3delta, reg4*) expression in the pancreas.

In NOD mice pancreas all *reg* genes were highly expressed and peaked at onset of disease (15 weeks). Only *reg 4* was not expressed.

Figure 25. Reg1 gene expression in the pancreas of NOD mice

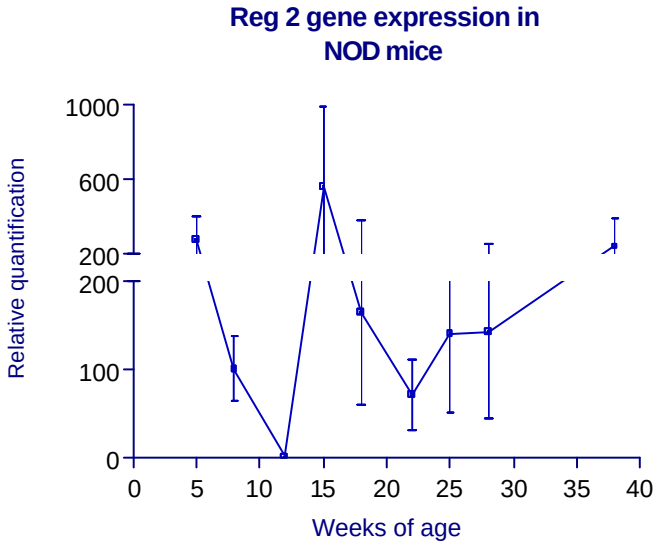


Figure 26. Reg2 gene expression in the pancreas of NOD mice

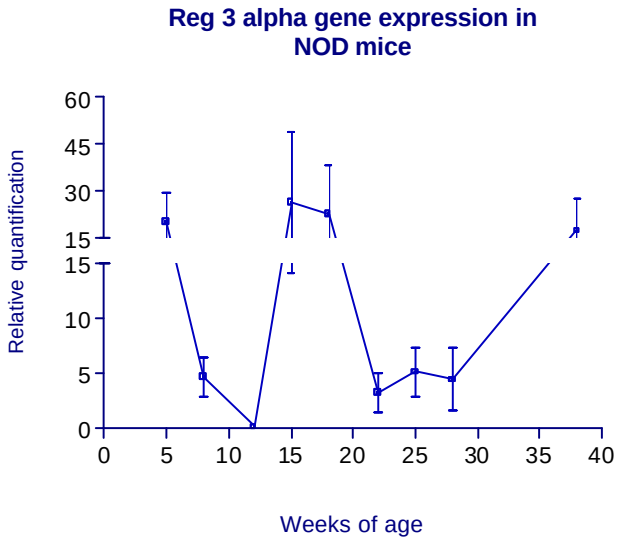


Figure 27. Reg 3alpha gene expression in the pancreas of NOD mice

Figure 28. Reg 3beta gene expression in the pancreas of NOD mice

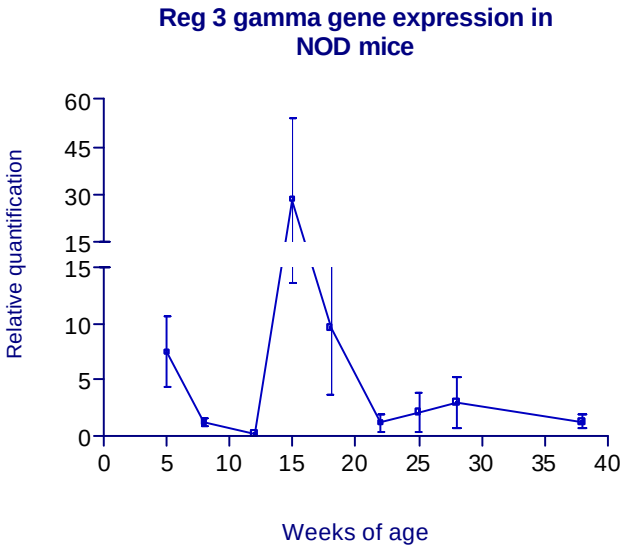


Figure 29. Reg 3gamma gene expression in the pancreas of NOD mice

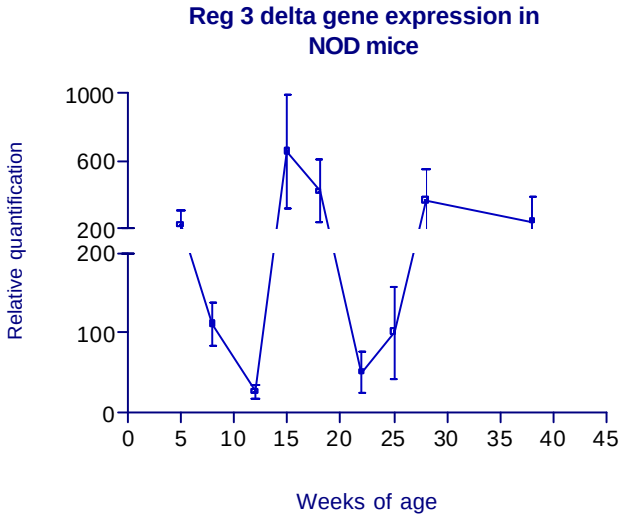


Figure 30. Reg 3delta gene expression in the pancreas of NOD mice

The same analysis was carried on BALB/C mice. Also these animals were sacrificed at diverse age. All Reg genes showed the same pattern: gene expression decreases dramatically from 4 weeks to 20 weeks.

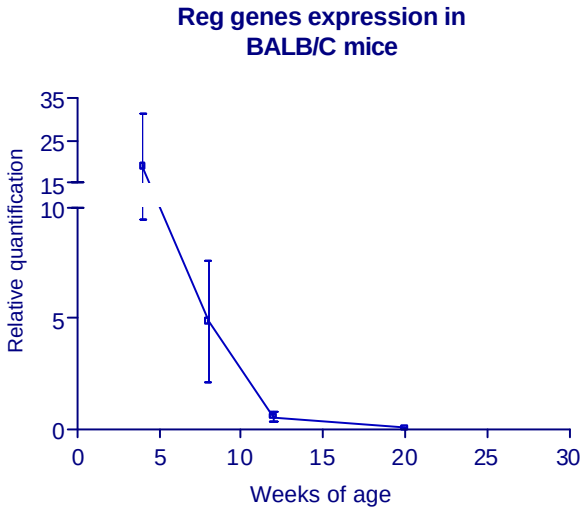


Figure 31. Reg genes expression in the pancreas of BALB/C mice

5.9 FUTURE WORKS

The data produced in this project are strong evidence of the presence of a regenerating environment in tissues that are being destroyed by autoimmunity. In future, additional proof of the mechanisms that drive this phenomenon and on how to modulate it needs to be achieved. Surely it will be necessary to increase the sample size to strengthen the significance of the research. More blood samples from T1D, SS, RA, SLE patients and control subjects should be collected, salivary glands and synovium samples from patients affected by SS and RA respectively and both NOD and control mice (BALB/C) need to be culled. All new samples,

human and murine, will be analysed as in the previous work and final data will be statistically evaluated.

Several components will be needed to carry out most of the future experiments, generating them will be a crucial part of the work.

From the genetic point of view, future studies will have to clarify the presence and role of new polymorphisms. We will search for polymorphisms in all autoimmune disorders studied: snips detection through DNA analysis of patients that show different degrees of regeneration (Reg protein levels in blood).

In the preliminary study only Reg 1alpha protein was dosed in patients affected by T1D, T2D, SS, RA, SLE and control subjects. Future work will evaluate all Reg proteins in the above subjects and also in patients affected by other autoimmune disorders such as Hashimoto's Thyroiditis, Grave's Disease, Crohn Disease and Multiple Sclerosis. These disorders in fact are autoimmune, chronic, inflammatory, organ specific pathologies and data from these other conditions will help understand Reg genes function.

According to the inflammatory molecules and their role in this project, several data need to be achieved. IL-6, Baff, April, Aid and a number of chemokines and cytokines levels will be dosed in T1D, SS, RA and SLE patients and control subjects.

Finally, cells will be cultured in the presence of IL-6 and Reg genes end protein expression/secretion will be evaluated before and after. The challenged cells will be studied to evaluate Reg genes expression, cytokine gene expression and production.

This program of work will try to elucidate the role of Reg in tissue destruction/regeneration as well as the potential relation of autoantibody production and disease state. With this work we will investigate the cellular and molecular mechanisms of autoimmunity to Reg genes and proteins in a large number of autoimmune disorders. This whole research will build enough knowledge and hopefully in a future project we will be able to explore Reg therapeutic potential (e.g. vaccines).

5.10 DISCUSSION

In the present study it has been demonstrated that REG genes are expressed in target inflamed tissues in autoimmune diseases. It has been demonstrated that in NOD mice pancreas all *reg* genes (*reg1, reg2, reg3alpha, reg3beta, reg3gamma, reg3delta*) are highly expressed and peak at onset of disease. These data are the strong evidence of a regenerating environment in tissues that are being destroyed by autoimmunity. According to the pancreas, the peak of REG genes expression at disease onset is an extremely relevant observation as it proves the attempt of the beta cells to re-establish their original mass when first assaulted by the immune system. These result give a clear observational base on which it will be possible to build functional studies to allow the molecular mechanisms underlying the phenomenon. The same analysis were carried on BALB/C mice. Also these animals were sacrificed at diverse age. All *reg* genes showed the same pattern: gene expression decreases dramatically from 4 weeks to 20 weeks. This data was never shown before in literature and is a very important observation

as it helps clarifying *reg* genes function in the pancreas of both NOD and healthy mice. We believe in fact that during the normal growth of the pancreas in early life, *reg* genes have an active role in inducing the physiological expanding process. Once the organ is formed, *reg* genes gradually decrease their degree of expression. In the NOD mice instead, *reg* genes are re-activated later in the animal life (12-15 weeks) consequentially to the organ damage. In fact, autoimmunity destroys beta cells and the loss activates *reg* genes at disease onset exactly as our RT-PCR data showed. The gene expression in NOD pancreas after disease onset decreases as an autoimmune process probably starts against Reg-secreting cells. Even if the *reg* genes expression in NOD decreases, in contrast to BALB/C, it never reduces to zero, not even after 30 weeks. BALB/C pancreas instead stop expressing *reg* genes once the organ has reached its adult volume.

Apart from the genetic observation, the results on the blood values of Reg 1alpha protein, are further evidence that cell regeneration occurs and starts after disease diagnosis. In this sense the assay of Reg1alpha may offer a novel tool to implement and monitor therapies for cell regeneration in some autoimmune diseases. Serum levels of Reg1alpha protein were measured in T1D subjects (31 newly diagnosed and 56 long standing), 63 T2D subjects, 40 SS patients, 39 RA patients, 39 SLE patients, 64 Controls sex/age matched (normal subjects).

Within the diabetic patients, each group showed a statistically significant difference with the control subjects. This observation supports the role of Reg 1alpha in diabetic pancreas. Regarding T1D, the lack of difference

between newly diagnosed and long standing patients (p=0.22) confirms the fact that Reg 1alpha is secreted at disease onset and implies the fact that disease evolution and therapy haven't got any effect on the protein secretion.

The prove that therapy, metabolic balance and residual beta cell function have no effect on the protein levels, can be found in the absence of correlation between C-peptide (marker of beta cell function), HbA1c (indicator of metabolic control), duration of disease, age and Reg1a ("possible" marker of beta cell regeneration) protein levels.

In this part of the study the genetic associations between Reg1alpha levels and T1D related genes polymorphisms (HLA, INS, PTPN22, CTLA4) was also evaluated but no association was found. This result confirms the idea that Reg genes expression is induced by the autoimmune disorder and is a consequent of the attempt of regeneration, not originally implicated in the disease pathogenesis.

Always within the diabetic patients, data on Reg 1alpha dosages showed a significant difference between T2D patients and controls (<0.001), newly diagnosed T1D and T2D patients (p=0.006) but no difference was present between long standing T1D and T2D patients (p=0.09). This results can be explained considering the role of inflammation at disease onset. Data confirmed by the NOD mice *reg* genes expression in the pancreas. It's possible to hypothesise that *reg* genes expression is induced by both cell death and inflammation at the beginning of the autoimmune process. At this stage in fact, both the cytokines and the regenerating need may be co-inducers of *reg* genes expression, determining the higher protein secretion

and explaining the higher Reg 1alpha levels found in newly diagnosed T1D patients. When all beta cells are destroyed by inflammatory cells, the infiltrates (insulitis) start to fade, and the few cells that regenerate from the precursors can be destroyed with a lower effort of the immune system, the levels of Reg proteins secreted are lower. At this stage in fact the immune system and inflammation aren't any more inducers of *reg* genes expression. This would explain the lower levels of Reg 1a in long standing T1D and T2D compared to T1D patients. This data is also sustained by the observation of the cytokine trend in pancreas of NOD mice: most of them peak at disease onset and than decrease.

Regarding the other autoimmune disorders that were studied in this work, significant difference was found between SS patients and controls ($p=0.0001$), RA patients and controls ($p=0.0001$), but no difference could be observed between SLE patients and controls. These interesting data can be explained with the hypothesis that the expression and secretion of Reg 1a protein is induced only in autoimmune diseases that affect a target organ and is not induced in systemic autoimmunity (SLE). To be able to give an explanation based on more evidence, this result needs to be correlated with other variables such as disease duration, therapy, cytokines levels in blood, autoantibodies production, histological data from salivary glans and synovium, eventual organ damage in SLE patients.

The presence of autoantibodies against Reg 1alpha in the sera, supports the idea that Reg proteins are new antigens secreted by regenerating organs and that the immune system activates against them. Even if this data encourages supporting the hypothesis of a vicious cycle between

autoimmunity and regeneration, still there is not enough evidence yet to carry on this explanation for the Reg genes mechanism of action.

The data achieved up to date together with the planned future work will provide enough evidence to fulfil the aims of this project consisting in the evaluation of the role of REG genes and proteins in cell regeneration in organs target of chronic inflammation in autoimmune diseases, and the assessment of the value of serum Reg proteins levels and Reg auto-antibodies as bio-marker of regeneration and disease respectively.

CHAPTER 6: REG GENES AND THE ACCELERATOR HYPOTHESIS

6.1 BACKGROUND

Diabetes is characterised by abnormal blood glucose levels which are controlled by two components: the beta cells which secrete insulin and the insulin sensitive tissues (liver, muscle, adipose) which respond to the insulin. Thus a failure in blood glucose control results either from failure of the beta cells to secrete insulin (T1D), resistance of the tissues to its action (T2D), or a combination of both[207]. The prevalence of diabetes of both types is increasing rapidly in industrialized countries and although much attention has focused on the increase in T2D, a parallel increase in T1D has been observed which also requires explanation[208]. Given that T1D is mainly an autoimmune disease and T2D an obesity and lifestyle related diabetes, the connection between diet and diabetes has traditionally focused on T2D. However, the distinctions between T1D and T2D are becoming increasingly blurred, both aetiologically and clinically[209].

6.2 THE ACCELERATOR HYPOTHESIS

The "Accelerator Hypothesis"[210] believes that body mass is central to the development and rising incidence of both types of diabetes. The control of weight gain and its associated insulin resistance could be the means of minimising the onset of T1D and T2D. More specifically, the Accelerator Hypothesis identifies three processes that variably accelerate the loss of beta cells through apoptosis, regardless of whether T1D or T2D,

constitution, insulin resistance and *autoimmunity*[211]. The Accelerator Hypothesis predicts an earlier onset in heavier people, without necessarily a change in risk, and views T1D and T2D as the same disorder of insulin resistance set against different genetic backgrounds[212]. None of the three accelerators leads to diabetes in the absence of weight gain, a trend which the hypothesis deems central to the rising incidence of all diabetes in the industrially developed and developing world. Weight gain causes an increase in insulin resistance, which results in the weakening of glucose control. Therefore, an increasing body weight in the industrialized world has been accompanied by earlier onset (i.e., acceleration) of diabetes.

The first accelerator, a constitutionally high rate of beta cell apoptosis, is necessary for diabetes to develop but in itself is rarely sufficient to cause it. **The second accelerator**, insulin resistance, results from weight gain and physical inactivity, which further increases the rate of beta cells apoptosis. Finally, a small and genetically defined subset of patients with both intrinsic lesions and insulin resistance, develop beta cell autoimmunity, which represents **the third accelerator**[213].

Of the three accelerators, one is intrinsic and two are acquired. Insulin resistance, the second accelerator, is associated with visceral fat mass and is widely believed to explain the epidemic rise of T2D in the industrially developed world. The Accelerator Hypothesis argues that visceral weight gain is also central to T1D, as much responsible for its rising incidence as

for that of T2D, and the environmental factor in T1D that has eluded epidemiology for so long.

The concept of an aetiological link between the two types of diabetes is not new but the evidence is now stronger. Rather than overlap between the two types of diabetes, the Accelerator Hypothesis envisages overlay. T1D is the same as T2D except for one essential add-on: immune response.

6.3 AIM OF THE STUDY

The overall aim of this study was to evaluate the effects of a reduced amount of food (4 gr. per day) instead of the "ad libitum" diet on the development of diabetes in the NOD mouse model.

Furthermore, with the present study, we tested the hypothesis that T1D can be delayed/prevented in the NOD mouse, by limiting weight gain (Accelerator Hypothesis) and therefore reducing the stress to the beta cells. A reduction in insulin production improves peripheral insulin sensitivity and also reduces the antigenicity of beta cells associated with its secretion.

The relationship between body weight and onset of diabetes has been examined in the context of weight at birth, weight changes since birth and weight at disease onset. Furthermore we tested the hypothesis that a diet with a lower protein and a greater starch amount could also impact disease development.

6.4 MATERIALS AND METHODS

48 female NOD mice were randomly allocated in two different groups:

group A) reduced food intake with 4 gr. per day using a high nutrient diet (RM3) group B) reduced food intake with 4 gr. per day using a diet poor in protein and rich in starch (RM1).

Mice were weighed regularly throughout their life span and tested weekly for urinary glucose (>56 mmol/l), as a sign of development of diabetes. At diabetes onset mice were removed from the experiment and their diabetic status confirmed by a blood-glucose reading of 11.5 mmol/l or greater.

During the study (at week 10,20, 30, 40, 50, 60, 70 and 80 of life/diet) 38 NOD non diabetic mice were removed from the initial project, were dissected and pancreas retained for studies aimed at expression of *reg* genes (*reg1, reg2, reg 3alpha, reg3beta, reg 3gamma, and reg 3delta*) associated with beta cell regeneration. RNA from pancreas was extracted and than reverse-transcribed into cDNA using the Thermoscript RT-PCR System. The real-time PCRs were than run in duplicate and all *reg* genes pancreatic expression was evaluated.

We analyzed mice exposed to different food availability- food restriction compared to the normal situation, in which mice have free access to food (*ad libitum)*- and how the age at onset of diabetes varies accordingly.

The normal "ad libitum" food intake for a NOD mouse is 5g per day. In the present project we have set up two different situations in relation to food intake:

1. Reduced food intake (4g per day)
2. Reduced intake of reduced calorie food (4g per day)

For group 1 we used the RM3 diet (Lilllico), which has an increased protein content and is the usual diet upon which the NOD colony is maintained. For group 2 we used the RM1 diet (Lillico), which is used as maintenance diet and is much lower in protein content. The rationale is to simulate the wild state where body mass is restricted by food availability. The food was divided in two rations to split energy release throughout the day.

Mice were singly housed since it was easier to monitor the mice once they are in separate cages and made the food intakes/ body weights more accurate.

Twenty-four female NOD mice were allocated to each group. This number is based on a power of 90% with a p-value (double sided t-test) of <0.05. The calculation of the sample size is based on the knowledge that these animals usually develop diabetes at 10-14 weeks and we expect that under this food restriction diabetes will develop at 20-22 weeks. By assuming a standard deviation of 6, the number of animals required is 22 for each group (24 animals per group will be included to allow for drop-outs).

Chiara Guglielmi

6.5 RESULTS

No significant difference was observed between group A and group B in terms of weight at birth (10.3 gr. ± 2 vs. 10.6 gr. ± 1.9), weight changes since birth considering the pre-diabetes phase (24.9 gr. ± 2.1 vs. 23.6 gr. ± 2.1) and weight at disease onset (23.7 gr. ± 2.1 vs. 21.5 gr. ± 3.5).

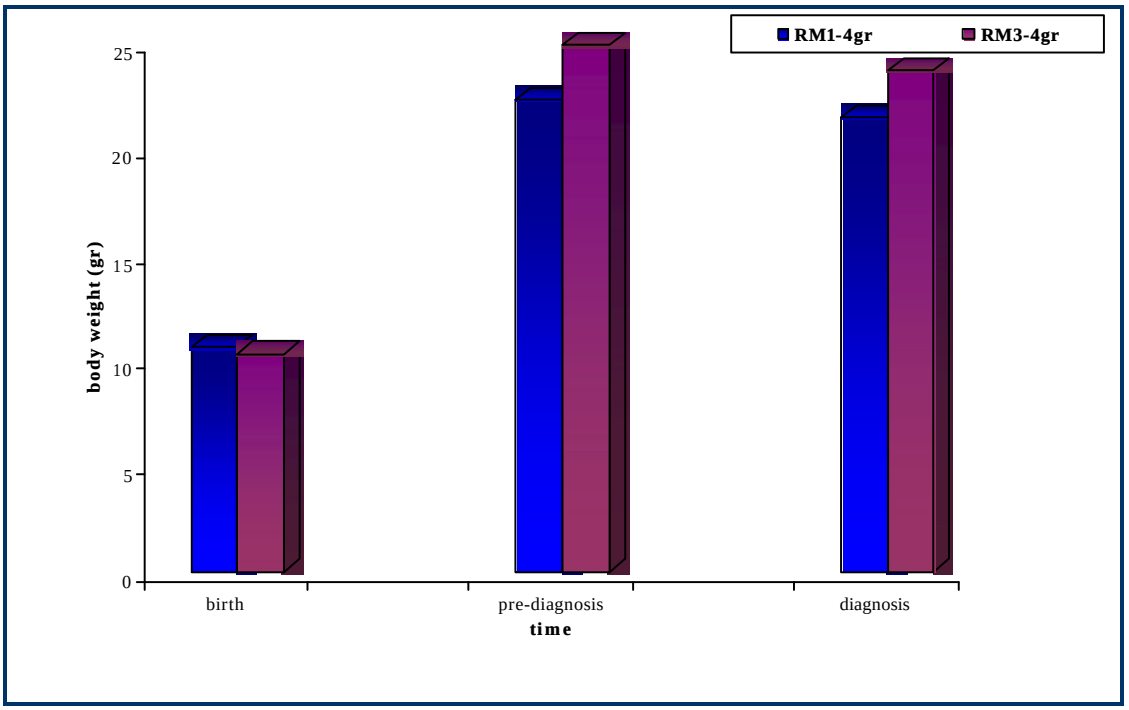


Figure 32. Body weight variations.

Incidence of diabetes was significantly reduced in group A (20.8 %) compared to group B (45.8 %, p=0.001).

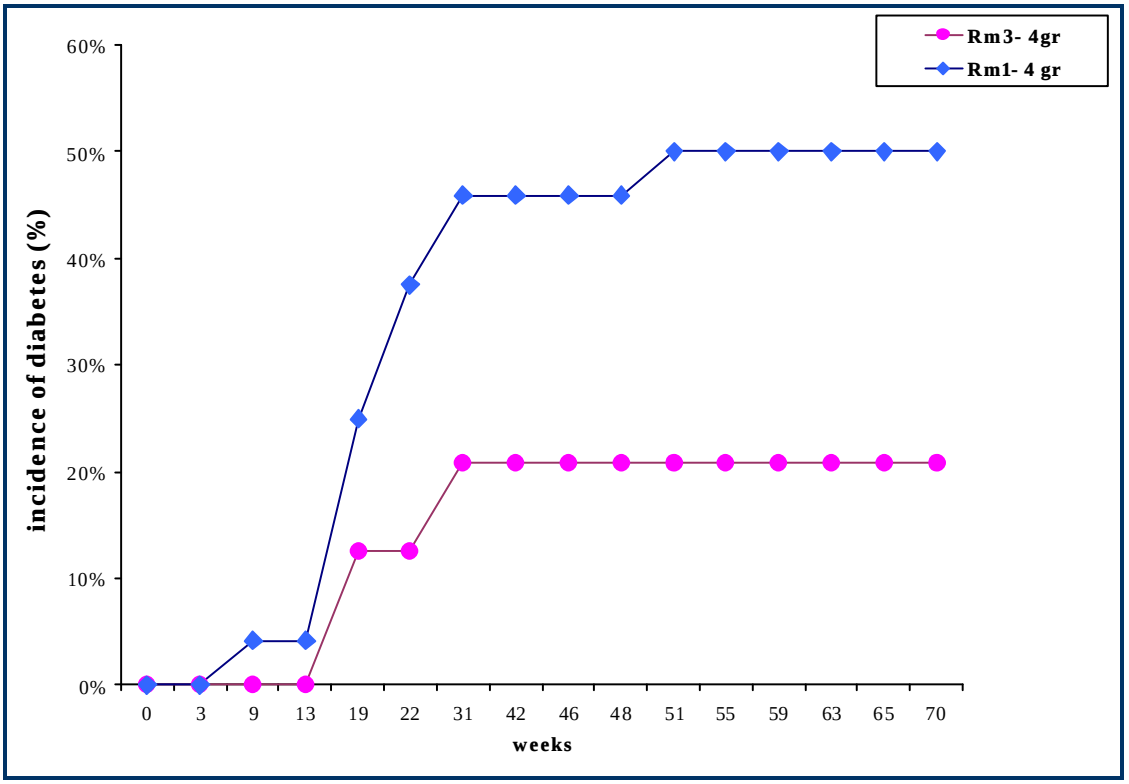


Figure 33. Incidence of diabetes in the two groups of study.

All *reg* genes were expressed in pancreas but a different trend in their expression between the two groups was observed.

The two groups of mice showed a different trend in all Reg genes expression.

This evidence may suggest:

1. a relation between *reg* genes expression and diet; after the RM3 diet, mice show an increased *reg* genes expression. The reduced glucose levels created by the diet (Accelerator Hypothesis) may create a permissive environment and allow an increase in *reg* genes expression (50 fold increase in RQ values). This observation may suggest that the diet and the low levels of glucose determine a reduction in the incidence of diabetes. At the same time induce the expression of *reg* genes.

2. Reg genes expression also showed a peculiar trend: expression of all *reg* genes increased until 30 weeks of age and then decreased in group A; on the contrary, in group B *reg* genes expression decreased first and then peaked up again at 30 weeks of age. This data concerning the trend of genes expression confirms the above hypothesis.

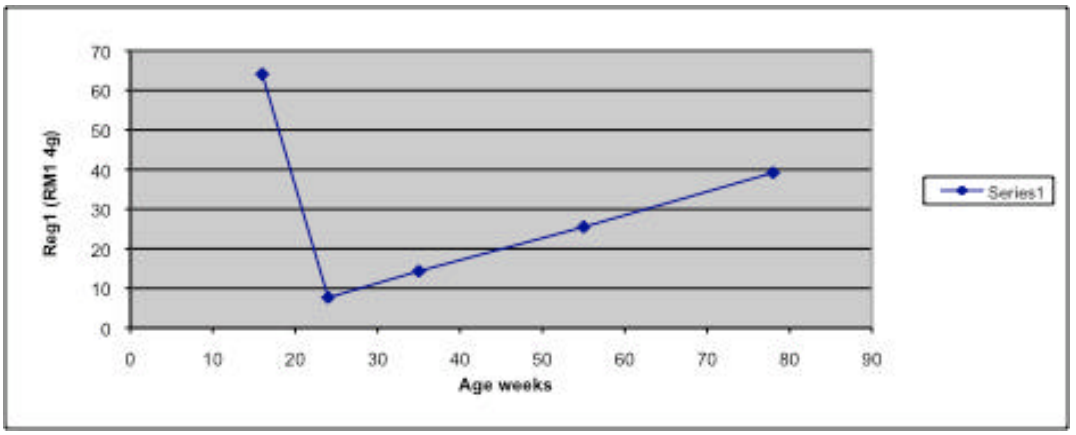


Figure 34. Reg1 gene expression in the pancreas of NOD mice feeded with diet RM1

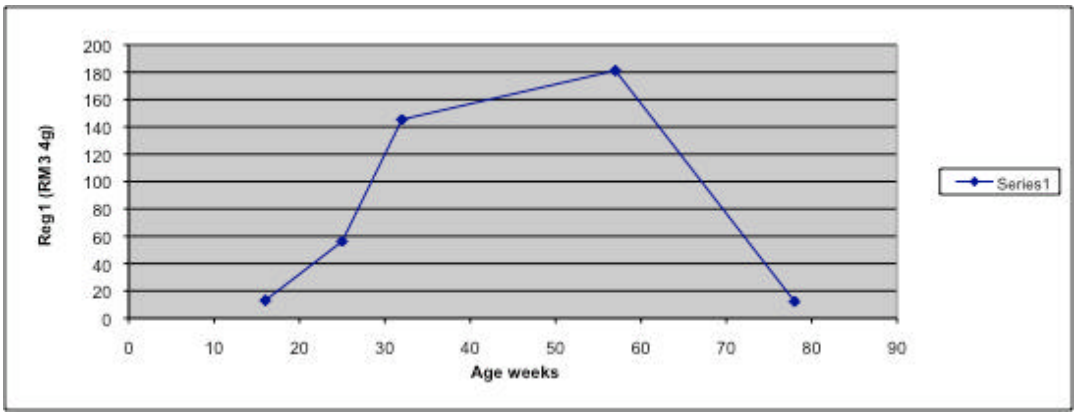


Figure 35. Reg1 gene expression in the pancreas of NOD mice feeded with diet RM3

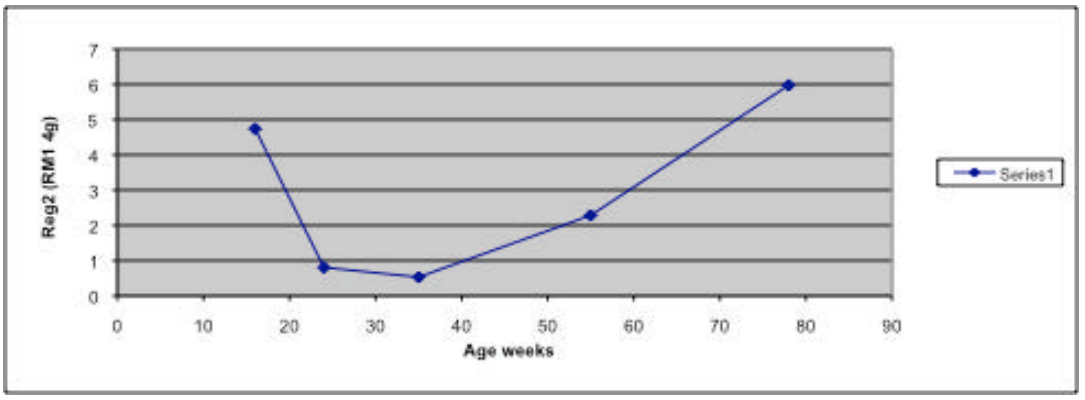


Figure 36. Reg2 gene expression in the pancreas of NOD mice feeded with diet RM1

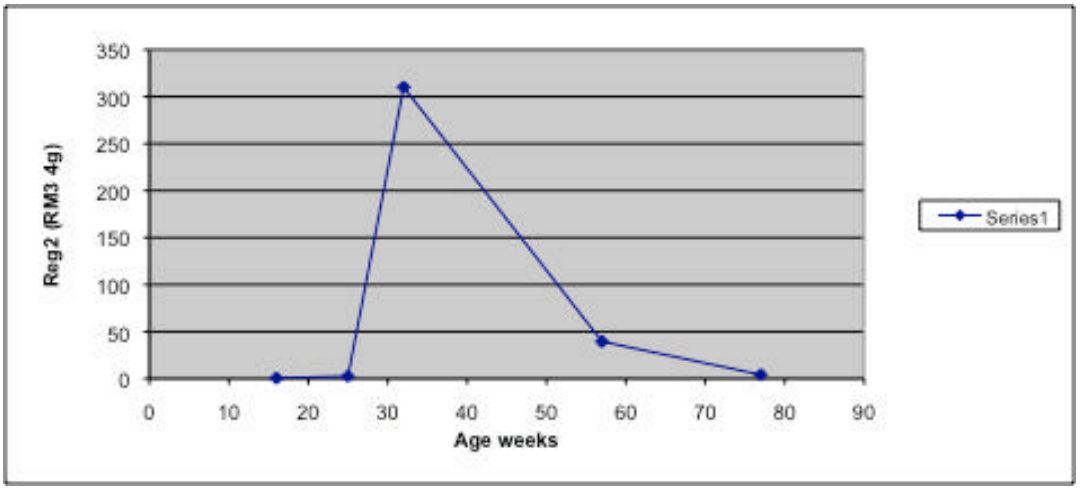


Figure 37. Reg2 gene expression in the pancreas of NOD mice feeded with diet RM3

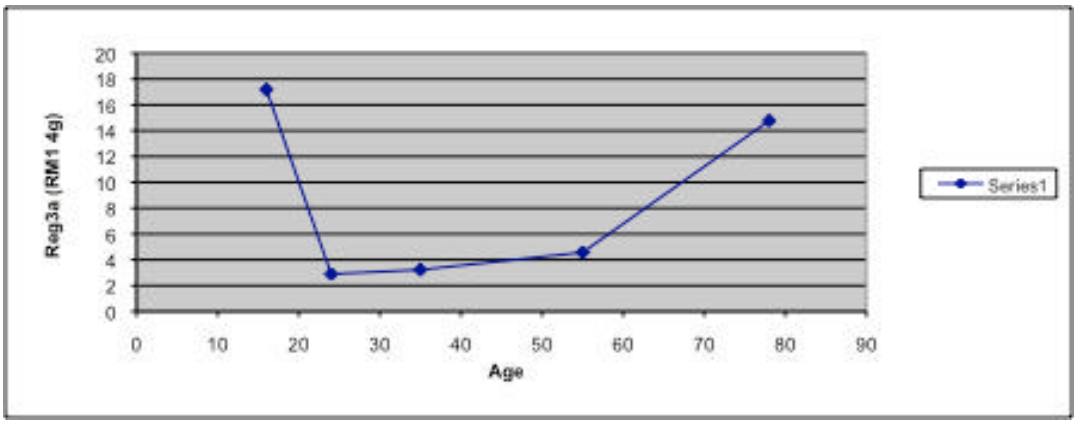


Figure 38. Reg3alpha gene expression in the pancreas of NOD mice feeded with diet RM1

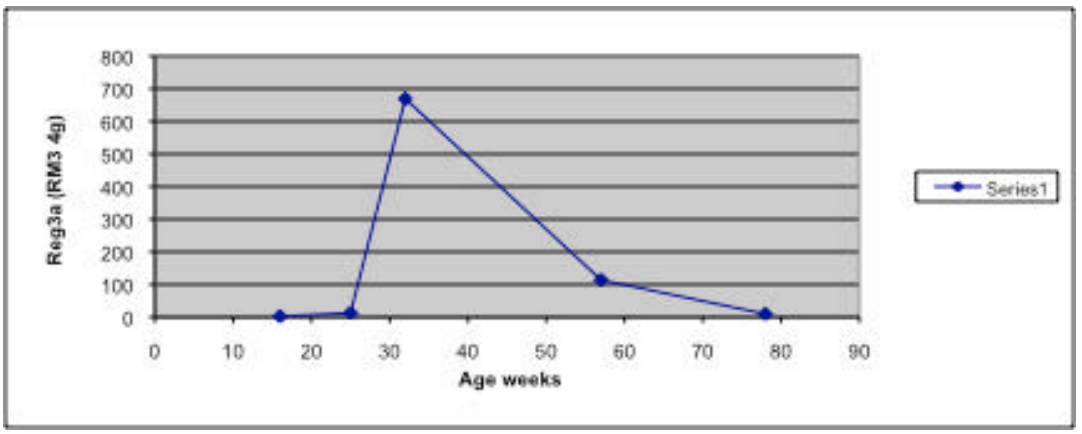


Figure 39. Reg3alpha gene expression in the pancreas of NOD mice feeded with diet RM3

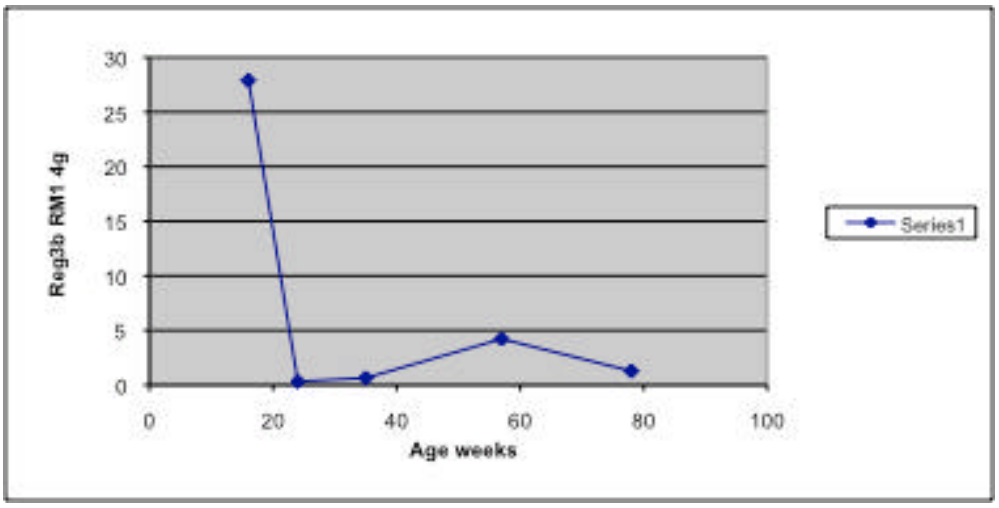


Figure 40. Reg3beta gene expression in the pancreas of NOD mice feeded with diet RM1

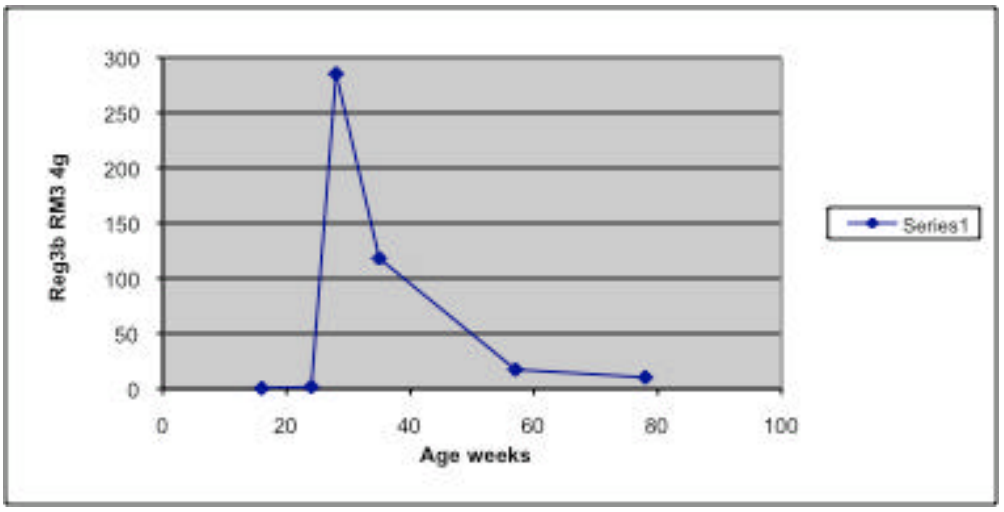


Figure 41. Reg3beta gene expression in the pancreas of NOD mice feeded with diet RM3

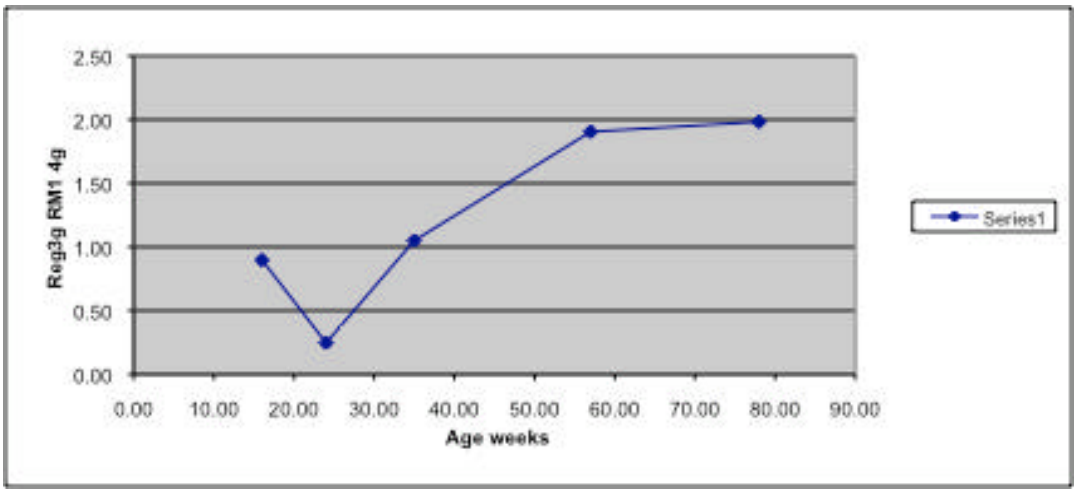


Figure 42. Reg3gamma gene expression in the pancreas of NOD mice feeded with diet RM1

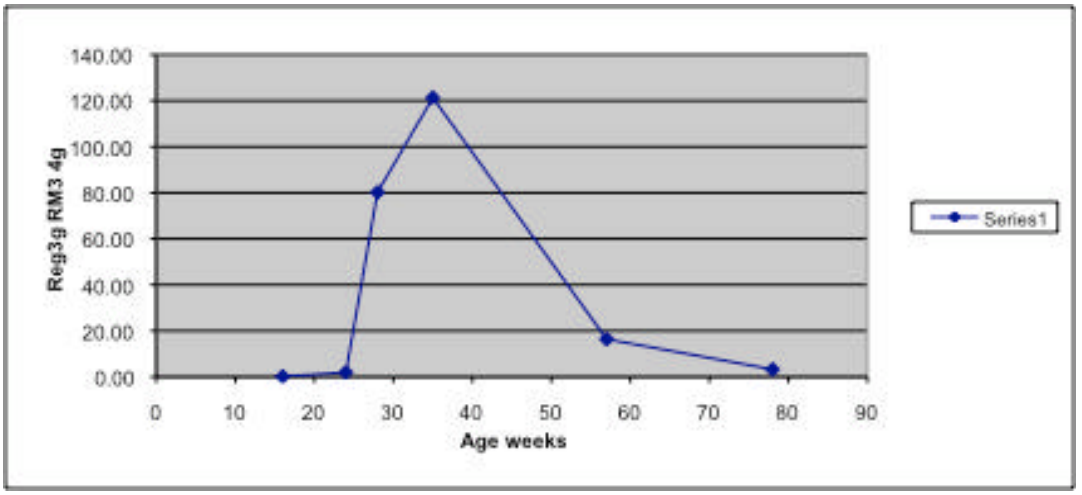


Figure 43. Reg3gamma gene expression in the pancreas of NOD mice feeded with diet RM3

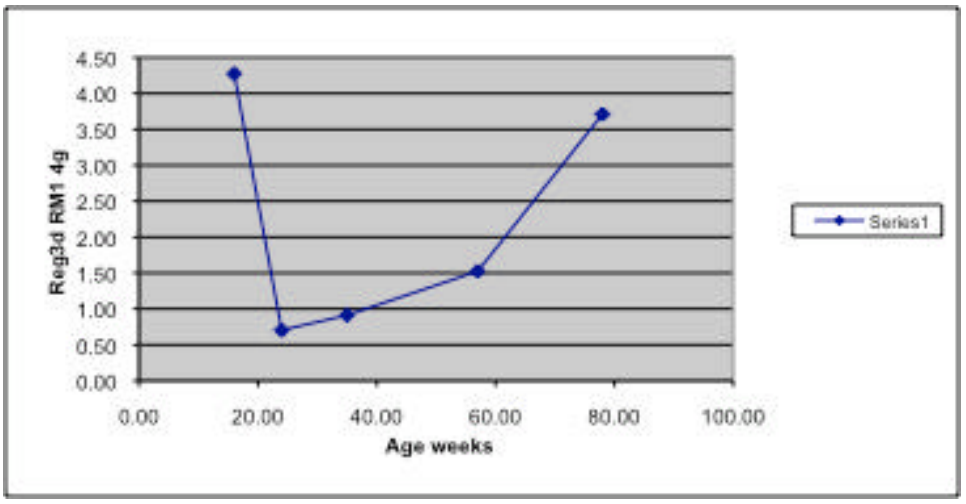


Figure 44. Reg3delta gene expression in the pancreas of NOD mice feeded with diet RM1

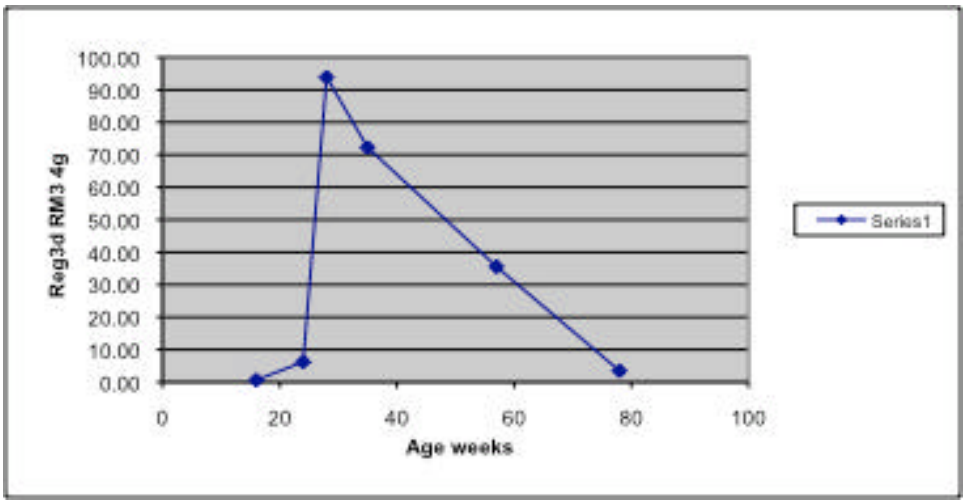


Figure 45. Reg3delta gene expression in the pancreas of NOD mice feeded with diet RM3

Chiara Guglielmi

Re: 44th EASD Annual Meeting, Rome, 7-11 September 2008

Abstract title: **A diet rich in protein and poor in starch with reduced food intake prevents diabetes in the NOD mouse: the significance of Reg gene expression in the pancreas**

June 2008

Dear Dr. Chiara Guglielmi,

The Programme Committee Meeting has been held from 28 – 30 May 2008.

It is our pleasure to inform you that your abstract

Submission-Number: *A-08-2301-EASD*
Abstract Title: **A diet rich in protein and poor in starch with reduced food intake prevents diabetes in the NOD mouse: the significance of Reg gene expression in the pancreas**

has been accepted as a **Poster Presentation** for the 44th EASD Annual Meeting which will be held in Rome, from 7 – 11 September 2008.

Within the next few days the official letter with more detailed information (presentation date, time, and instructions, etc.) will be sent to you by e-mail and post.

If you have not yet registered, please be reminded that the **early registration deadline is 20 June 2008**. Online Registration is available on the EASD website: http://www.easd.org.

Yours sincerely,

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6.6 FUTURE WORKS

From diabetic mice we collected tissues (pancreas, liver and spleen) and now we we are planning to carry out some mechanistic studies.

We will investigate the following parameters to find out the mechanisms involved in the protective effect of diet restriction:

a) ***the insulitis process***, by measuring the type of lymphocytes infiltrating the islets, in order to investigate the influence of diet restriction on the homing of committed T-cells. These histological studies will be perfomed on sections cut from the pancreas by staining with haemotaxylin/eosin.

b) ***the apoptosis process***, by calculating numbers of apoptotic cells in the islets of each of the 3 groups of mice we can directly investigate whether diet restriction reduces the rate of programmed cell death. These histological studies will be performed on sections cut from the pancreas, and apoptosis will be calculated following staining with haemotaxylin/eosin, and differences in apoptotic cells calculated for each group. Apoptotic cells are detected by the presence of morphological characteristics such as condensed nuclei, membrane blebbing and cellular fragmentation. This study will further be supported by staining tissue sections for Caspase-3 antibody. Caspase-3 is an effector in the apoptotic pathway and is activated just prior to the formation of apoptotic bodies.

c) ***the insulin content***, by extraction from the pancreas of the NOD mice after the exposure to different diets to confirm that mice which have eaten more have produced more insulin; INSIK-5 RIA kits will be used to assay insulin content from the supernatant.

CHAPTER 7: FINAL REMARKS

T1D is an autoimmune disease accounting for more than 80% of all cases of diabetes in children and adolescents. The incidence of T1D is rising worldwide and the cost of diabetes and its complications exceed $100 billion annually.

T1D results from selective immune mediated destruction of pancreatic beta cells and strategies to prevent or reverse the development of diabetes can be divided into three groups, depending on whether they focus on beta cell protection, beta cell regeneration or beta cell replacement.

The regeneration of beta cells for T1D is a longstanding research goal. New ways to regenerate beta cells destroyed by autoimmune disease have been generated by means of the explosion of interest in stem cells. While most research attention has been on transplanting stem cells from exogenous sources, many other regenerative therapies are emerging to harness the body's endogenous sources — a strategy with inherently lower risks of immune rejection.

Cell replacement therapies are potentially promising approaches to treating several diseases, including diabetes, Parkinson's, Alzheimer's and other autoimmunity related diseases among others.

Besides stem cell derived islets for reversing T1D, other approaches such as in vivo trans-differentiation and regeneration are becoming more attractive.

In conclusion, it is feasible to protect and to regenerate beta cells in those patients with T1D and this endeavour should be further pursued.

Our efforts must however be address also to the induction of the immunological tolerance in order to avoid new reactivation of the autoimmune process once generated new beta cells.

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