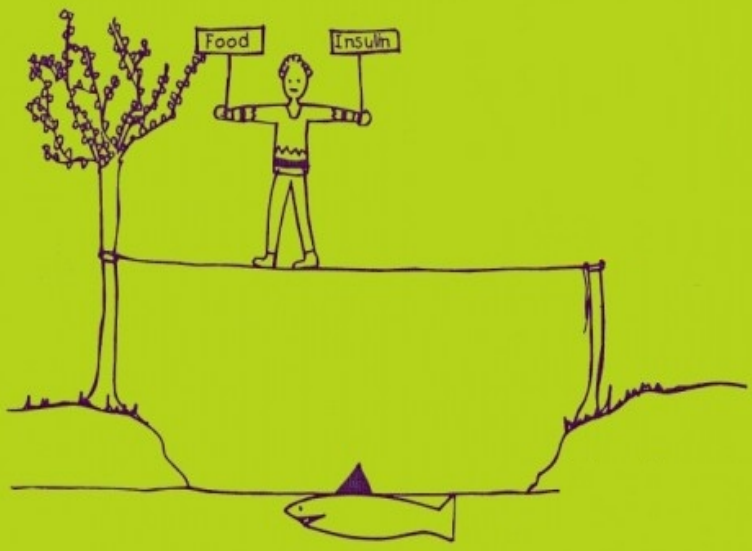
Type 1 Diabetes COMPLICATIONS



Edited by **David Wagner**

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Contents

Preface

This book is a compilation that includes reviews on type 1 diabetes onset, complications of cardio, vascular, retinal, oral health and potential treatment options. Authors have reviewed current literature on each of these topics to provide an excellent compendium on current understanding of how type 1 diabetes evolves and progresses with more emphasis on diabetic complications and on the current status of treatment strategies.

The etiology of diabetes remains a mystery. There is discussion about the genetic predisposition and more detailed complications including neural, nephropathy and co-morbidity in youth. The autoimmune nature of the disease including CD4+ and CD8+ T cells have been extensively explored; yet why these cells become pathogenic and the underlying causes of pathogenesis are not fully understood. This book is an excellent review of the most current understanding on development of disease with focus on diabetes complications.

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Part 1

Diabetes Onset

1

Genetic Determinants of Microvascular Complications in Type 1 Diabetes

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1. Introduction

Diabetes mellitus is one of the most prevalent chronic diseases of modern societies and a major health problem in nearly all countries. Its prevalence has risen sharply worldwide during the past few decades (Amos et al., 1997; Shaw et al., 2010). Moreover, predictions show that diabetes prevalence will continue to rise, reaching epidemic proportions by 2030: 7.7% of world population, representing 439 million adults worldwide (Shaw et al., 2010). This increase is largely due to the epidemic of obesity and consequent type 2 diabetes (T2DM). However, the incidence of type 1 diabetes (T1DM) is also rising all over the world (DiaMond Project Group, 2006; Maahs et al., 2010). Recent data for Europe (Patterson et al., 2009) predict the doubling of new cases of T1DM between 2005 and 2020 in children younger than 5 years and an increase of 70% in children younger than 15 years, old.

Despite major progresses in T1DM treatment during the past decades, mortality in T1DM patients continues to be much higher than in general population, with wide variations in mortality rates between countries. In Europe, these variations are not explained by the country T1DM incidence rate or its gross domestic product, but are greatly influenced by the presence of its chronic complications, especially diabetic renal disease (Groop et al., 2009; Patterson et al., 2007). In fact, much of the health burden related to T1DM is created by its chronic vascular complications, involving both large (macrovascular) and small (microvascular) blood vessels.

Many genetic, metabolic and hemodynamic factors are involved in the genesis of diabetic vascular complications. However, major epidemiological and interventional studies showed that chronic hyperglycemia is the main contributor to diabetic tissue damage (DCCT Research Group, 1993). If the degree of metabolic control remains the main risk factor for the development of diabetic chronic complications, an important contribution can be attributed to genetic risk factors, some of them common for all microvascular complications (diabetic retinopathy, neuropathy, and renal disease) and some specific for each of them (Cimponeriu et al., 2010). Additional factors are represented by some accelerators such as hypertension and dyslipidemia.

In the following pages, we present briefly the pathogenesis type 1 diabetes and its chronic microvascular complications. The main information of the genetic background in T1DM with particular focus on gene variants having strong impact on endothelial dysfunction as the key factor in the development of microvascular disorders are also summarized.

2. Type 1 diabetes mellitus

T1DM is a common, chronic, autoimmune disease characterized by the selective destruction of the insulin secreting pancreatic beta cells, destruction mediated mainly by the T lymphocytes (Eisenbarth, 1986). The destruction of the insulin secreting pancreatic beta cells is progressive, leading to an absolute insulin deficiency and the need for exogenous insulin treatment for survival. The pathogenic factors that trigger anti beta cell autoimmunity in genetically predisposed subjects are not yet fully elucidated, but there is clear evidence that it appears consequently to an alteration of the immune regulation. The destruction of the beta cells in T1DM is massive and specific and it is associated with some local (Gepts, 1965) or systemic (Bottazzo et al., 1978) evidence of anti-islet autoimmunity.

It is currently considered that, on the "background" of genetic predisposition, some putative environmental trigger factors will initiate the autoimmune process that will finally lead to T1DM. Identifying these triggers proved to be difficult, mainly due to a long period of time elapses between the intervention of the putative environmental trigger and the clinical onset of overt diabetes. The most important factors seem to be non-genetic external (environmental) ones. However, from environmental factors repeatedly associated with T1DM, the most important were viral infections, dietary and nutritional factors, nitrates and nitrosamines, etc. (Akerblom et al., 2002).

Genetic factors in the pathogenesis of T1DM in humans. T1DM is a common, complex, polygenic disease, with many predisposing or protective gene variants, interacting with each other in generating the global genetic disease risk (Todd, 1991). The study of candidate genes identified several susceptible genes for T1DM (Concannon et al., 2009): IDDM1 encoded in the HLA region of the major histocompatibility complex (MHC) genes on chromosome 6p21 and genes mapped to the *DRB1, DQB1* and *DQA1* loci , IDDM2 encoded by the insulin gene on chromosome 11p15.5 and mapped to the *VNTR* 5′ region, IDDM12 encoded by the cytotoxic T lymphocite associated antigen 4 (*CTLA4*) gene on chromosome 2q33, the lymphoid tyrosine phosphatase 22 (*PTPN22*) gene on chromosome 1p13 and the *IL2RA/CD25* gene on chromosome 10p15. The genome wide linkage (GWL) analysis strategies (Morahan et al., 2011) or genome wide association (GWA) techniques (Todd et al., 2007) led to the identification of other T1DM associated loci, for most of which the causal genes are still not elucidated.

3. Chronic complications in T1DM

T1DM is characterized by the slow progression towards the generation of some specific lesions of the blood vessels walls, affecting both small arterioles and capillaries (microangiopathy) and large arteries (macroangiopathy). The "classical" diabetes microvascular complications are represented by *diabetic retinopathy* (DR) the main cause of blindness, *diabetic nephropathy* (DN) also known as renal disease, the main cause of renal substitution therapy (dialysis or renal transplantation) in developed countries, and *diabetic neuropathy* (DPN) as reported (IDF, 2009). As we already mentioned, chronic hyperglycemia represents the key determinant in the development of T1DM chronic microvascular complications. Meanwhile, considerable biochemical and clinical evidence (Hadi & Suwaidi, 2007) indicated that endothelial dysfunction is a critical part of the pathogenesis of vascular complications both in T1DM and T2DM.

Several mechanisms explain the contribution of chronic hyperglycemia to the development of endothelial dysfunction and chronic diabetes complications. An unifying mechanism was proposed by Michael Brownlee, suggesting that overproduction of superoxide anion (O_2^-) by the mitochondrial electron transport chain might be the key element (Brownlee, 2005). According to this theory, hyperglycemia determines increased mitochondrial production of reactive oxygen species (ROS). Increased oxidative stress induces nuclear DNA strand breaks that, in turn, activate the enzyme poly ADN-ribose polymerase (PARP) leading to a cascade process that finally activates the four major pathways of diabetic complications: (1) *Increased aldose reductase* activity and activation of the *polyol pathway* lead to increased sorbitol accumulation with osmotic effects, NADPH depletion and decreased bioavailability of nitric oxide (NO). (2) *Activation of protein kinase C* with subsequent activation of NF-kB pathway and superoxide-producing enzymes. (3) *Advanced glycation end-products* (AGEs) generation with alteration in the structure and function of both intracellular and plasma proteins. (4) *Activation of the hexosamine pathway* leads to a decrease in endothelial NO synthase (NOS3) activity as well as an increase in the transcription of the transforming growth factor (TGF-β) and the plasminogen activator inhibitor-1 (PAI-1) as reported (Brownlee, 2005).
In Europe, the prevalence of DN was estimated at 31%, DR was diagnosed in 35.9% of patients while proliferative DR in 10.3%. Apart hyperglycemia, the most important risk factor was the duration of the disease. Thus, the prevalence of proliferative DR is null before 10 years diabetes duration but 40% after 30 years duration while the prevalence of DN is null before 5 years diabetes duration but reaches 40% after 15 years of diabetes (EURODIAB IDDM Complications Study Group, 1994). Similar data were provided by the diabetes control and complications trial (DCCT) study in USA. Thus, after 30 years of diabetes, the cumulative incidence of proliferative DR and DN was 50% and 25%, respectively, in the DCCT conventional treatment group (DCCT/EDIC Research Group et al., 2009).

3.1 Diabetic nephropathy

DN in T1DM can be defined by the presence of increased urinary albumin excretion rate (UAER) on at least two distinct occasions separated by 3–6 months (Mogensen, 2000). DN is usually accompanied by hypertension, progressive rise in proteinuria, and decline in renal function. According to several guidelines, normal UAER is defined as an excretion rate below 30 mg/24 h; microalbuminuria represents an UAER between 30-300 mg/24 h while more than 300 mg/24 h defines overt proteinuria. In T1DM, five DN stages have been proposed (Mogensen, 2000). Stage 1 is characterized by renal hypertrophy and hyperfiltration, being frequently reversible with good metabolic control. Stage 2 is typically asymptomatic and lasts for an average of 10 years. Typical histological abnormalities include diffuse thickening of the glomerular and tubular basement membranes as well as glomerular hypertrophy. About 30% of subjects will progress towards microalbuminuria. Stage 3 (incipient DN) develops 10 years after the onset of diabetes. Microalbuminuria, the earliest clinically detectable sign, is well correlated with histological findings of nodular glomerulosclerosis. About 80% of subjects will progress to overt proteinuria. This proportion may decrease with tight glycemic control, hypoproteic diet and early treatment with angiotensin I-converting enzyme (ACE) inhibitors or angiotensin II receptor (Ang II R) blockers. Stage 4 (clinical or late DN) occurs on average 15–20 years after diabetes onset and is characterized by macroalbuminuria. The glomerular filtration rate (GFR) declines progressively, UAER increases usually to more than 500 mg/day and blood pressure starts

to rise. Histologically, mesangial expansion develops, renal fibrosis becomes more evident and leads to diffuse and nodular glomerulosclerosis. Stage 5 (end-stage renal disease) occurs on average 7 years after the development of persistent proteinuria. GFR decreases below 40 ml/min and an advanced destruction of all renal structures is observed.

DN pathogenesis is very complex and comprises both metabolic and haemodynamic factors in the renal microcirculation (Stehouwer, 2000). The glucose dependent pathways were presented briefly above. Haemodynamic factors mediate renal injury via effects on systemic hypertension, intraglomerular haemodynamics or via direct effects on renal production of cytokines, such as TGFβ and vascular endothelial growth factor (VEGF), or hormones such as angiotensin II or endothelin (ET) as reported (Schrijvers et al., 2004). In addition to the diabetes duration reflected by the level of glycated hemoglobin (HbA1c), the specific risk factors for DN are the blood pressure, older age, male sex, smoking status, and ethnic background.

Despite clear evidence for the role of genetic factors in DN, success in identifying the responsible genetic variants has been limited due to both objective and subjective difficulties, the main being represented by the small size of the DNA collections available to individual research groups (Pezzolesi et al., 2009). Strategies for the genetic investigation of DN included the analysis of candidate gene polymorphisms in case-control settings (hypothesis driven approach) as well as the GWL or GWA strategies with DN (hypothesis free approach). Numerous candidate genes were tested explaining the complexity of the diabetic renal disease pathogenesis (Cimponeriu et al., 2010; Mooyaart et al., 2011) but just few of them were reconfirmed in multiple, independent, studies. We give a list of the stronger associations in Table 1.

Gene	Chromo some	SNP	Allele	No. studies	Case/ Control	OR
ACE	17q23	*rs179975*	*D*	14	2215/2685	1.13
AKR1B1	7q35	*(AC)n repeat*	*Z-2*	10	1380/1308	1.12
		(AC)n repeat	*Z+2*	10	1380/1308	0.79
		rs759853	*T*	4	636/537	1.58
APOC1	19q13.2	*rs4420638*	*G*	2	857/935	1.54
APOE	19q13.2	*E2/E3/E4*	*E2*	6	889/803	1.48
EPO	7q21	*rs1617640*	*T*	2	1244/715	0.67
GREM1	15q13-q15	*rs1129456*	*T*	2	859/940	1.53
HSPG2	1p36.1	*rs3767140*	*G*	2	417/240	0.64
NOS3	7q36	*rs3138808*	*a*-del 393 bp	3	679/657	1.45
		rs2070744	*C*	2	273/450	1.39
UNC13B	9	*rs13293564*	*T*	4	1572/1910	1.23
VEGFA	6p12	*rs833061*	*C*	2	242/301	0.48

Table 1. Gene variants associated with DN in T1DM subjects. Identified by candidate gene study and confirmed after meta-analysis of at least 2 studies (adapted from a recent report, Mooyaart et al., 2011). I-converting *ACE*, angiotensin I-converting enzyme; *AKR1B1*, aldose reductase; *APOC1*, apoprotein C1; *APOE*, apoprotein E; *EPO*, erythropoietin; *GREM1*, gremlin 1 homolog; *HSPG2*, heparan sulfate proteoglycan; *NOS3*, endothelial nitric oxide synthase; *UNC13B*, presynaptic protein; *VEGFA*, vascular endotehlial growth factor A.

3.2 Diabetic retinopathy

DR is one of the most severe diabetes complications, potentially leading to severe sight decrease or even blindness. The first clinical signs of DR (incipient, non-proliferative DN) are retinal microaneurysms, dot intraretinal hemorrhages and hard exudates (Frank, 2004). The most severe stage, proliferative DR, is characterized by retinal haemorrhages from fragile neo-vessels and, in advanced eye disease by vitreous hemorrhages and tractional detachments of the retina, both resulting in visual loss. Histologically, DR is characterized by a selective loss of pericytes from the retinal capillaries followed by the loss of capillary endothelial cells (Frank, 2004). DR pathogenesis is complex, involving both metabolic and haemodynamic factors. The most important DR specific pathways seem to be the local production of several polypeptide growth factors, including VEGF, pigment-epithelium–derived factor (PEDF), growth hormone and insulin-like growth factor-1 , as well as cytokines and inflammatory mediators such as TNFα, TNFβ, TGFβ and NO (Frank, 2004). Genetic factors appear also to have an important role in generating the DR risk in T1DM subjects with similar degrees of metabolic control and disease duration (Keenan et al., 2007). The most often studied DR candidate genes include blood pressure regulators (RAS), metabolism factors (AKR1B1, AGER, GLUT1), growth factors (VEGF, PEDF), NOS2A, NOS3, TNFα, TGFβ, ET-1 and its receptors, etc. (Cimponeriu et al., 2010; Ng, 2010). As for all diabetes chronic complications, the studies of candidate genes were more often underpowered to detect true associations, and most often the results were not reconfirmed by additional, independent studies. However, published meta-analyses suggest a real role in DR for at least four genes (Abhary et al., 2009a; Cimponeriu et al., 2010; Ng, 2010).

Similar with DN, *ACE* gene was the most studied for DR in T1DM and data regarding its involvement will be presented further (see subchapter 6.1). *VEGFA* gene on chromosome 6p12-p21 was also intensively studied and a recent large study (including both T1DM and T2DM cases) suggested a possible effect of two gene variants (*rs699946* and *rs833068*) on DR risk in T1DM subjects (Abhary et al., 2009b). Among the candidate genes from the oxidative stress/increased ROS pathway, *NOS3* gene was intensively studied in DR and data will be presented in subchapter 4.3. Finally, maybe the strongest evidence for a role in the genetic risk for DR is provided by the analysis of *AKR1B1* gene on chromosome 7q35, encoding the rate-limiting enzyme of the polyol pathway. The most intensively studied polymorphism was the *(AC)n* microsatellite located at 2.1 kb upstream of the transcription start site (*Z-2*, *Z* and *Z+2* alleles). A recent meta-analysis (Abhary et al., 2009a) showed that the *Z-2* allele of the *(CA)n* microsatellite is significantly associated with DR risk in both T1DM and T2DM subjects. In addition, the *T* allele of *rs759853* in the *AKR1B1* promoter seems to be protective. To our best knowledge, no attempts for both GWL and GWA for identification of DR genes in T1DM were reported so far.

3.3 Diabetic polyneuropathy

DPN is a chronic microvascular complication affecting both somatic and autonomic peripheral nerves. It may be defined as the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes, after the exclusion of other causes of neuropathy. Many neuropathic patients have signs of neurological dysfunction upon clinical examination, but have no symptoms at all (negative symptoms neuropathy). On the contrary, some patients have positive symptoms (burning, itching, freezing, sometimes intense pain and often with nocturnal exacerbations), usually with distal onset and proximal

progression. This form is designated as painful DPN. Correct estimates regarding the prevalence of DPN are hard to obtain since the diagnosis of the "negative symptom patients" can be made only by active screening, usually with complex investigations such as nerve conduction velocity.

It is generally accepted that DPN results from the micro-angiopathy damage of the *vasa nervorum* (responsible for the microcirculation of neural tissue) associated with the direct damage of neuronal components induced by various metabolic factors, the most important being chronic hyperglycemia (Kempler, 2002). The vascular and metabolic mechanisms act simultaneously and have an additive effect. The most important links between the two are represented by the local NO depletion and failure of antioxidant protection, both resulting in increased oxidative stress. Apart the unquestionable role of chronic hyperglycemia (diabetes duration and level of metabolic control), other risk factors for DPN are increasing age, cigarette smoking, alcohol or other drug abuse, hypertension and hypercholesterolemia.

Data regarding the genetic background of DPN are rather scarce. To our best knowledge, no GWL or GWA were performed for the identification of DPN genes in T1DM. Several data regarding the effect of some candidate genes were published, but these included usually only small number of patients/controls and few were replicated in other, independent datasets. Maybe the most significant effect on DPN genetic risk in T1DM is conferred by variants of the *AKR1B1* gene. Other significant but not reconfirmed associations with the risk for DPN were reported for variants of *PARP-1*, *NOS2A, NOS3*, uncoupling protein *UCP2* and *UCP3,* genes encoding the antioxidant proteins, catalase and the superoxide dismutase, and the gene encoding the neuronal Na^+/K^+-ATPase (Cimponeriu et al., 2010).

In conclusion, during the past two decades we have witnessed an explosion of studies regarding the genetic background of diabetes microvascular complications, both in T1DM and T2DM. These efforts, mainly focusing on candidate genes and often using study groups underpowered to detect genuine associations, have contributed to the identification of a few credible predisposing gene variants (Doria, 2010). In order to make significant progresses in elucidating the genetics of microvascular complications, there is an urgent need for assembling large population collections of different backgrounds for both GWA scanning and candidate gene association studies.

4. Nitric oxide synthase genes

NO is one of the vasodilatory substances released by the endothelium and has the crucial role in vascular physiopathology including regulation of vascular tone and blood pressure, hemostasis of fibrinolysis, and proliferation of vascular smooth muscle cells (SMC). In T1DM, NO has an increased stimulatory effect on the released insulin from β cells, mostly to the early phase of the effect of glucose upon insulin secretion. Abnormality in its production and action can cause endothelial dysfunction leading to increased susceptibility to hypertension, hypercholesterolemia, diabetes mellitus, thrombosis and cerebrovascular disease. Serum nitrite and nitrate (NOx) concentrations assessed as an index of NO production was used as a marker for endothelial function.

In DN, the NO production was significantly higher. A strong link between circulating NO, glomerular hyperfiltration, and microalbuminuria in young T1DM patients with early nephropathy was reported (Chiarelli et al., 2000). It has been postulated that in diabetic kidney there is increased NO synthase (NOS) activity, and the excessive NO production can

induce the renal hyperfiltration and hyperperfusion and by its perturbing effect contributes to the DN appearance (Bazzaz et al., 2010). In early diabetes, the retinal circulation devoided of any extrinsic innervation and depending entirely on endothelium-mediated autoregulation, is dramatically affected by the ECs dysfunction due to the lack of the local NO, a state seen in DR (Qidwai & Jamal, 2010).

In active progressive DR, aqueous NO levels are significantly high, while plasma NO levels remained at the level of diabetics without DR (Yilmaz et al., 2000). Raised plasma NO levels in T1DM patients were reported (Heltianu et al., 2008) indicating that pathogenesis of diabetic-associated vascular complications is connected with a generalized increased synthesis of NO throughout the body. This phenomenon occurs early in the natural course of diabetes and independently of the presence of microvascular complications. So, we suggest that the high NO levels found in diabetic patients (including those without any clinically manifested microangiopaties) might represent an overproduction of NO that is associated with diffuse endothelial dysfunction (Heltianu et al., 2008).

There is a family of NOS enzymes which produces NO. The two constitutive isoforms NOS1 (neuronal) and NOS3 (endothelial) as well as the inducible isoform NOS2 have similar enzymatic mechanisms but are encoded on separate chromosomes by different genes. The *NOS1* gene is located on chromosome 12q24.2-24.31, has 29 exons, spaning a region greater than 240 kb and encodes a protein of ~161 kDa. The *NOS2A* gene is on chromosome 17q11.2–12 having 27 exons, spaning 37 kb and encodes a protein of ~131 kDA (Li et al., 2007). *NOS3* gene is on chromosome 7q35-36, includes 26 exons, having a genomic size of 21 kb, and encodes a protein of ~133 kDa (Chen et al., 2007; http://www.genecards.org, version 3).

4.1 *NOS1* gene

The NOS1-derived NO is implicated in local regulation of vascular tone and blood flow using different mechanisms. This process appears to be independent of central NOS1 action on autonomic function (Melikian et al., 2009). In the early stages of diabetes, the NOS1 expression in the nitrergic axons decreases and its level in the cell bodies is unaffected probably due to a defect in axonal transport. Insulin treatment is able to reverse NOS1 decrease. With the progression of diabetes, NOS1 accumulates in the cell bodies due to an affected transport down to the axons, and the degenerative changes become irreversible without any response to insulin treatment (Cellek, 2004). The *NOS1* gene has 12 different potential first exons (*1A–1L*) and as consequence the NOS1 protein is expressed as a very complex enzyme (Wang et al., 1999). The NOS1B is expressed in renal microvasculature (Freedman et al., 2000). To our knowledge, there are only two reports in which *NOS1* polymorphisms were analyzed for the relationship with diabetic microvascular disorders. Microsatellite markers in *NOS1B* were assessed in T2DM and an association with ESDR for alleles 7 and 9 was reported (Freedman et al., 2000). The *CA* repeat in the 3'-UTR region (exon 29) of *NOS1* was found not to be a risk for DPN (Zotova et al., 2005).

4.2 *NOS2A* gene

NOS2A gene has the transcription start site in exon 2 and the stop codon in exon 27. This gene encodes NOS2 protein which has two different functional catalytic enzyme domains, the oxygenase domain encoded by 1 to 13 exons, and reductase domain by 14 to 27 exons. The NOS2 differs from the constitutive forms (NOS1 and NOS3) being Ca^{2+} independent.

Due to strong binding of calmodulin to NOS2, this is insensitive to changes in calcium ion concentrations (Jonannesen et al., 2001; Qidwai & Jamal, 2010). Under normal conditions, NOS2 is not expressed. Exposure to high ambient glucose or cytokines, the upregulation of NOS2 occurs in a variety of cell type and tissues. As a consequence, a sudden burst of NO synthesis occurs leading to severe vasodilation and circulatory collapse. In diabetic milieu as long as NOS3 expression is low, the induction of NOS2 expression may occur in an attempt to achieve homeostasis, being crucial in preventing or delaying pathological alterations in the microcirculation (Warpeha & Chakravarthy 2003). In studies of diabetic complications, as DR and DN, influenced by vascular functional disturbances, the increased NO formation via NOS2 expression has been reported (Johannesen et al., 2000a).

In human *NOS2A* gene has been identified a large number of polymorphisms. In the promoter region there are single nucleotide polymorphisms (*-954G/C*, *-1173C/T*, *-1659 A/T*) and two microsatellite repeats, the biallelic *(TAAA)*n, and the *(CCTTT)*n with nine alleles, which might affect the *NOS2* transcription. Explanations for this modulation was proposed for *–1173C/T* polymorphism, when the *C* to *T* change predicts the formation of a new sequence recognition site for the GATA-1 or GATA-2 transcription factors, which further bind to specific DNA sequences and potentially increase the degree of mRNA transcription (Qidwai & Jamal, 2010). The gene variants in the coding region might alter the activity of *NOS2* with subsequence variability in the NO levels which might be responsible for the susceptibility or/and severity of the disease. Other polymorphisms in exons and introns were reported, *rs16966563* (exon 4, *Pro68Pro*), *rs1137933* (exon 10, *Asp385Asp*), *rs2297518* (exon 16, *Leu608Ser*), *rs3794763* (intron 5, *G>A*), *rs17718148* (intron 11, *C>T*), *rs2314809* (intron 17, *T>C*), and *rs2297512* (intron 20, *A>G*).

In T1DM, the *NOS2A* polymorphisms in the promoter region [*-954G/C*, (*TAAA*)n and (*CCTTT*)n] and exons (*Asp346Asp* and *Leu608Ser*) were analyzed and the results showed that none of them has a role in the development of the disease (Johannesen et al., 2000a). Using the transmission disequilibrium test, it was found in Caucasian population that there is an increased risk for T1DM among *HLA DR3/4*-positive individuals with a *T* in position 150 in exon 16 (*Leu608Ser*) of *NOS2A*. This finding suggests an interaction between the *NOS2A* locus and the HLA region and a role for *NOS2A* in the pathogenesis of human T1DM (Johannesen et al., 2001).

Assessement of polymorphisms in T1DM for the prevalence of DR showed that the *14-repeat* allele of (*CCTTT*)*n repeat* polymorphism in *NOS2A* was significantly associated with the absence of the disease. A person with diabetes carrying this allele has 0.21-fold chance of developing retinopathy as compared to those not carrying the allele, suggesting that the carriage of the *14-repeat* allele is not a feature of diabetes itself, but is specific to DR development (Warpeha et al., 1999; Warpeha & Chakravarthy, 2003). In addition, the same *NOS2A* variant, the *14-repeat* allele, was found to represent a low risk for DN (Johannesen et al., 2000b), and other report indicated that carriers of this allele have the low risk of DPN in T1DM (Nosikov, 2004; Zotova et al., 2005).

4.3 *NOS3* gene

NOS3 is the most relevant and frequent isoform studied to assess the role of genetic issues in the development of angiopathic disease in T1DM. This enzyme is a constitutively expressed in vascular endothelial cells, and the protein expression depends on Ca^{2+} and calmodulin. It was suggested a possible dual functionality of NO. Excessive production of

NO, in DN, induces the renal hyperfiltration and hyperperfusion and contributes to the vascular disorder. More often, reduced NO production or availability was reported in other vascular pathologies. The effect of NO on the endothelial modulation is influenced by the duration of diabetes; so, at early stages of diabetes the endothelial function is enhanced, and with the progression of diabetic duration the endothelial dysfunction is accelerated (Bazzaz et al., 2010; Chen et al., 2007; Mamoulakis et al., 2009).

Several polymorphisms have been reported in NOS3 promoter, exon and intron regions (Table 2). The most studied variant from the promoter region was the single nucleotide polymorphism at position -786 where there is a base substitution from *T* to *C* (*rs2070744*). In previous studies it was shown that individuals with *−786C* allele had a reduced activity of the *NOS3* gene promoter (Taverna et al., 2005), explained by the fact that DNA binding protein (replication protein A1) has the ability to bind only to the *−786C* allele resulting a ~50% reduced *NOS3* transcription, with the subsequent decrease in both protein expression and serum NOx levels (Erbs et al., 2003). The interrelationships among *rs2070744* genotypes, NOS3 (mRNA, protein levels, and enzymatic activity), and plasma NOx levels have never been linear.

NOS3 polymorphism in intron 4 (*4a/4b*) is based on a variable 27-base pair tandem repeat four (allele *4a*), five (allele *4b*) or six (allele *4c*) repeats. Previous studies have suggested that deletion of one of the five nucleotide repeats in intron 4 could affect the rates of *NOS3* transcription and processing rate, thus resulting the modulation of NOS3 enzymatic activity and, apparently, affecting the plasma NOx concentrations (Zanchi et al., 2000), with the potentiality of this genotype to have an effect on microangiopathy later on in diabetic life (Mamoulakis et al., 2009).

SNP ID	Chromosome position	Location type	Alleles
		intron 4	*4b/4ba*
743507	150707488	intron	*A/G*
1799983	150696111	exon	*G/T*
1800783	150689397	intron	*T/A*
2070744	150690079	the 5′ promoter region	*T/C*
2373929	150345745	the 3′ region	*G/A*
2373961	150312143	the 5′ promoter region	*C/T*
3138808		Del/Ins 393 bp	*D/I*
3918188	150702781	intron	*A/C/T*
12703107	150314562	the 5′ region	*G/T*
41322052	150690106	intron	*C/T*

Table 2. *NOS3* gene polymorphisms. Source, http://www.genecards.org; version 3

Carriers of the *4a* allele were found exhibiting ~20% lower NOx levels that appearing in *4b/4b* homozygous subjects. The regulation of *NOS3* expression is more complicated considering the strong linked of *4a/4b* variant with *rs2373961* and *rs2070744* when the *b/b* genotype might acts independently and in coordination with the other variants (Chen et al., 2007; Zintzaras et al., 2009). Among polymorphisms found in exons of *NOS3*, the *G* to *T* polymorphism at position 894 in exon 7 (*rs1799983*) was most studied. It was reported that

this variant changes the NOS3 protein sequence, probable resulting an alteration of enzyme activity (Costacou et al., 2006), and control the NOS3 intracellular distribution interacting with proteins of degradating process (Brouet et al., 2001).

From many polymorphisms of the *NOS3* gene some of them are associated with the development of diabetic microvascular complications while others indicated their protective role (Freedman et al., 2007; Heltianu et al., 2009). A recent study of *rs2070744* in Caucasian T1DM reported a positive association with diabetes *per se* as well as DR and two possible explanations were found; either *NOS3* is a candidate gene for the microvascular disease, or there is a linkage disequilibrium between *NOS3* and the neighbouring genes. It is known that in the same position (7q35) to *NOS3* gene the AKR1B1 and T-cell receptor beta-chain (TCRBC) genes in the 7q34 position are located (Bazzaz et al., 2010). In a hyperglycaemic milieu, the retinal NO bioavailability due to the presence of C-786 mutant allele of rs2070744 is decreased, and therefore the lack of NO stimulates aldose reductase, known to be implicated in the development of diabetes complications (Chandra et al., 2002). Other report showed that the onset pattern of severe DR in longstanding C-peptide-negative T1DM is affected by *NOS3 rs2070744* and *C774T* polymorphisms (Taverna et al., 2005). In the case of *C774T NOS3* polymorphism, the association with severe DR was related to the influence of the DN presence, which is a well-known strong risk factor for DR (Cimponeriu et al., 2010). Oppose, the rare allele *4a* of *4b/4a* variant of *NOS3* was found to be related to absent or non-severe DR in T1DM Caucasians patients, suggesting a protective role. Although the *4b* allele was more frequent among patients with severe DR, a modest effect on the microvascular disorder was evaluated from the broad confidence interval (Cheng et al., 2007). Recent reports and our studies showed that there were no relationships between *4b/4a* variant of NOS3 and DR or other microangiopathic complications. Similar results for *rs1799983* in relation with DR were also reported (Heltianu et al., 2009; Mamulakis et al., 2009). In a meta-analysis of genetic association studies for DR in T1DM, from the three *NOS3* polymorphisms (*rs1799983*, *rs3138808* and *rs41322052*) included in the sub analysis for Caucasian subjects, none of them were found to be significantly associated with any form of DN (Abhary et al., 2009a)

The progression of renal disease was associated with the *NOS3 rs2070744* variant (Freedman et al., 2007; Zanchi et al., 2000), a result confirm recently by meta-analysis (Mooyaart et al., 2011; Ned et al., 2010). Contradictory results were obtained for the relationship of *NOS3 4b/4a* polymorphism with DN. Some reports showed no association (Degen et al., 2001; Heltianu et al., 2009) and others indicated that the *4a* allele represents an excess risk for advanced DN (Nosikov, 2004; Zanchi et al., 2000; Zinzaras et al., 2009). It was hypothesized that the *NOS3 4b/4a* itself plays a role in tissue-specific regulation of *NOS3* expression, a mechanism related to the importance of intron structure in the splicing of immature to mature RNA or to the presence of enhancer sequences within the intron 4. On the other hand, both *rs2070744* and *4b/4a* polymorphisms were specifically associated with advanced DN, and the *-786C/4a* haplotype was reported to be transmitted from heterozygous parents to siblings with advanced DN, suggesting that the *4a* allele is coupled almost exclusively with the *-786C* allele of *rs2070744* (Zanchi et al., 2000). The *NOS3 rs1799983* was analyzed in T1DM Caucazians from different countries and some reports showed no association with DN (Heltianu et al., 2009; Möllsten et al., 2009; Nosikov, 2004) and others found a marginal relationship (Ned et al., 2010) or strong association with increased risk of DN (Zintzaras et al., 2009). The *-786C/894T* haplotype of *NOS3* was found to be significantly associated with

albuminuria, suggesting a strong implication of this gene in the susceptibility to kidney damage (Ned et al., 2010). The *rs3138808* variant of *NOS3* was also analyzed in a meta-analysis and was found to be associated with DN (Mooyaart et al., 2011).
There are only few reports which analyze the influence of *NOS3* polymorphisms on DPN in T1DM. Data from Caucasian patients genotyped for *rs1799983* and *4b/4a* variants showed that both polymorphisms were not associated with DPN (Nosikov, 2004; Zotova et al., 2005**).** Our findings showed that only *NOS3 4b/4a* was not associated with DPN (Heltianu et al., 2009). In T1DM subjects with the lowest incidence of confirmed DPN, it was reported that the *894G* carriers of *rs1799983* variant had fivefold increased risk for DPN, suggesting that despite low risk for the disease in these individuals, there is a genetic predisposition to develop diabetes-related complication (Costacou et al., 2006). In agreement with this report we found a prevalence of DPN among the *894GG* as compared with *894TT* homozygotes in diabetic patients with normal kidney function, suggesting that *894GG* genotype might be a risk factor for T1DM-related microvascular disease. This subgroup of DPN patients with *894GG* had over 42% DR as an additional vascular complication, and the presence or absence of DR did not modify the significance of the relationship between the *rs1799983* polymorphism and DPN. In addition, these subjects were recorded with high systolic blood pressure and raised levels of NOx, indicating a possible endothelial dysfunction, as well as with high levels of triglycerides, suggesting that additional high risk lipid profile contribute to the aggravation of the microvascular disorder. We presume that the rare-type *894T* allele might have a protective role against the development of DPN and a tendency to counterbalance increased NO production due to both chronic hyperglycemia and hypoxic effect at the microvascular level, by a not yet elucidated, compensatory-type mechanism (Heltianu et al., 2009).
Taken together, these results indicate that in T1DM, from various *NOS3* polymorphisms the most studied were *rs2070744*, *rs1799983* and *4b/4a* variants. Even in Caucasians there are differences among populations for the effects of gene polymorphisms on the microvascular complications. Diverse factors contribute to the variations between studies, analysis of early or late microvascular complication, incidence of the studied disorder in subjects with other confirmed disease, small sample size, the lack of haplotype analysis. Further studies on larger numbers of samples and on different populations are required to confirm these results.

5. Endothelin genes

The family of endothelins (ET) is represented by three peptides (1 to 3) and two receptors (ETRA and ETRB), which are widely distributed, in different proportions, being mostly abundant in vascular endothelial cells (EC). Their ET-1 and ET-2 are strong vasoconstrictors, whereas ET-3 is a potentially weaker vasoconstrictor compared to the other two isoforms. The ET-1 which is the most potent vasoconstrictor peptide acts as a paracrine or autocrine factor and its effects are ~10 times higher that of angiotensin II. The ET-1 has a variety of functions including its significant contribution to the maintenance of basal vascular tone, modulation of vascular permeability for proinflammatory mediators and proliferation of SMC. Having a long half-life, only a slight activation of its receptors into the signaling pathways might contribute to progressive disturbances, as hypertension and diabetic microvascular disorders (Cimponeriu et al., 2010). From the two receptors, the ETRA, expressed in SMC, has the highest affinity for ET-1, and is involved in the short term

regulation of SMC and in the long term control of cell growth, adhesion and migration in the vasculature. The ETRB, expressed on both EC and SMC, has a dual function and can cause both vasoconstriction on SMC and vasodilation by the release of endothelial NO (Kalani, 2008; Potenza et al., 2009). The components of ET family are encoded by different genes (Table 3) with a generic name *EDN* (*EDN1*, *EDN2*, *EDN3*, *EDNRA*, and *EDNRB*). All three *EDN* genes (1 to 3) translate a respective amino acid prepropeptide, which is cleaved by one or more dibasic pair-specific endopeptidases to yield big ET. For ET-1, the large precursor is then converted into the mature and active ET-1 by a putative converting enzyme (ECE-1) encoded by the *ECE1* gene.

In diabetes, the secreted ET-1 by kidney cells activates its receptors and leads to constriction of renal vessels, inhibition of salt and water reabsorption, and enhanced glomerular proliferation. Correlations between plasma or urinary levels of ET-1 and signs of DN at different stages, as well as a close association between systemic endothelial dysfunction and microalbuminuria have been reported. Elevated ET-1 levels are present before the onset of microalbuminuria in T1DM, and worsen in association with it. In DR, the increased ET-1 levels strongly correlate with the enhanced endothelial permeability and loss of endothelial-mediated vasodilation in the retinal microvasculature (Kalani, 2008; Kankova et al., 2001). In DNP, the ET-1 is a potent vasoconstrictor of vasa nervorum and contributes to the EC abnormalities, when the balance of vasodilatation and vasoconstriction is in the favor of the latter. Moreover, ETA receptors contribute to the development of peripheral neuropathy, while ETB receptors have a protective role (Kalani, 2008; Lam, 2001). Most of the reported findings were for T2DM. A difference in the ET-1 involvement in the development of microvascular disorders in T1DM can not be excluded, knowing that differences in the pathogenesis of microangiopathy between type 1 and type 2 diabetes might exist.

Gene		Protein			
Name	Location	Name	Size		Subcellular location
			a.a.	kDa	
EDN1	6p24.1	ET-1	212	24.43	secreted
EDN2	1p34.2	ET-2	178	19.96	secreted
EDN3	20q13.2-13.3	ET-3	238	25.45	secreted
ECE1	1p36.1	ECE1	770	87.16	cell membrane
EDNRA	4q31.22	ETA receptor	427	48.72	cell membrane
EDNRB	13q22	ETB receptor	442	49.64	cell membrane

Table 3. The endothelin family. Source, http://www.genecards.org; a.a., amino acids; kDa, kiloDalton

The *EDN1* gene has different polymorphisms including the *-3A/-4A*, a *−138* insertion/deletion and the *CA/CT* dinucleotide repeat in promoter, the *C8002T* or TaqI variant in intron 4, and the *Lys198Asn*, a *G/T* polymorphism in exon 5. In *EDNRA* gene were reported the *−231 A/G* and *C1363T* variants, while in *EDNRB* gene the *A30G* polymorphism. Assessments of relationship between variability of plasma concentrations of ET-1 (and big ET-1) and *EDN1* polymorphisms (*G8002A* and *−3A/−4A*) in patients with chronic heart failure indicated that there was no significant association, suggesting that the genetic

variants are not risk factors, but plasma ET-1 level influences more the disease severity (Spinarová et al., 2008).

Insufficient data exists regarding the influence of *EDN1* polymorphisms on the development of microvascular disorders. In a previous review was shown that *EDN1* gene was directly involved in hypertension and polymorphisms in *EDNRA* were associated with essential hypertension testifying the necessity of a balance within the endothelin system for normal functioning in vascular tissues. Although the importance of ET-1 expression in retinal microvasculature in high glucose was incontrovertible, it appears to be a lack of association between *EDN1* and *ECE1* polymorphisms and DR (Warpeha & Chakravarthy, 2003). Interestigly, in T2DM the *TT* genotype of *EDN1 G/T* polymorphism was associated with reduce risk of DN (Li et al., 2008).

6. Genes of renin – Angiotensin system

Renin-angiotensin system plays a central role in blood pressure regulation and fluid electrolyte balance, being a modulator of vascular tone and structure. RAS components are produced by different organs and are delivered to their site of action by the bloodstream. Angiotensinogen (ANGT) is synthesized primarily by the liver and the released hormone precursor is cleaved by renin enzyme and aspartyl proteinase, to generate angiotensin I (Ang I). The key enzyme of RAS is angiotensin I-converting enzyme (ACE) which converts Ang I to angiotensin II (Ang II) by the release of the terminal His-Leu, when an increase of the vasoconstrictor activity of angiotensin occurs. The Ang II acts through two main receptors, the type 1 Ang II receptor and the type 2 Ang II receptor (Table 4). It is generally believed that type I Ang II receptor is the dominant one in the cardiovascular system, being expressed in different organs including the brain, kidney, heart, skeletal muscle (Abdollahi et al., 2005).

Gene		Protein			
Name	Location	Name	Size		Subcellular location
			a.a.	kDa	
ACE	17q23.3	ACE	1306	149.72	secreted and cell membrane
ACE2	Xp22	ACE2	805	92.46	secreted and cell membrane
AGT	1q42-q43	ANGT	485	53.15	secreted
AGTR1	3q24	Type 1 Ang II receptor	359	41.06	cell membrane
AGTR2	Xq22-q23	Type 2 Ang II receptor	363	41.18	cell membrane

Table 4. Renin-angiotensin system. Source, http://www.genecards.org; a. a., amino acids; kDa, kiloDalton

The RAS effects are primarily mediated by Ang II, a trophic hormone, which acts either directly on tissues, including vascular remodeling and inflammation or indirectly on NO bioavailability and its consequences (Chung et al., 2010; Ringel et al., 1997). In distinct local organs (brain, kidney, eye, vessel wall, heart) RAS regulatory mechanisms and function are

different, so the Ang II actions may be modulated by a specific physiological process of a given tissue system. A variety of stimuli, including hyperglycemia, hypertension, sodium intake, inflammation modulate the expression of the tissue RAS components in pathophysiological states, and chronic production of Ang II may proceed remodeling and restructuring in various cardiovascular organs (Conen et al., 2008).

Discovery of ACE homologue, angiotensin I-converting enzyme 2 (ACE2) increased the complexity of RAS. ACE2 is predominantly expressed in endothelium of different tissues (i.e. kidney), although its distribution is much less widespread than ACE. The enzyme hydrolyses different peptides, including Ang I and Ang II, and is implicated in hypertension, diabetic nephropathy, and cardiovascular disease (Fröjdö et al., 2005). ACE2 seems to act as a negative regulator of the RAS, counterbalancing the function of ACE thus promoting vasodilation (Giunti et al., 2006).

RAS is a causative factor in diabetic microvascular complications inducing a variety of tissue responses including vasoconstriction, inflammation, oxidative stress, cell hypertrophy and proliferation, angiogenesis and fibrosis. Most of previous reports showed the RAS role in the initiation and progression of diabetic nephropathy. In the kidney, Ang II affects renal hemodynamics, tubular transport and stimulates growth and proto-oncogenes in various renal cell types. Increased production of angiotensin II within nephrons and their vasculature could participate in the local renal injury through both hemodynamic and nonhemodynamic actions and is a well-established factor promoting renal damage (Gumprecht et al., 2000). Because the low conversion of Ang I in the kidney, it has been proposed that the plasma ACE circulating through the kidney is an important contributor but yet a limiting factor in angiotensin II production within the renal circulation (Marre et al., 1997).

On the other hand, ACE2 which has a similar distribution to ACE, being largely localized in renal tubules, when is downregulated, as in diabetes-associated kidney disease, leads to an increase of tubular Ang II, which, in turn, may promote tubulointerstitial fibrosis. In early phases of diabetes in the absence of renal injury, it was suggested that ACE2 expression is increased, and in compensation the ACE was inhibited preventing the diabetes associated renal disease. These findings suggest that ACE inhibition may confer a renoprotective effect (Giunti et al., 2006). In diabetes, damage to the retina occurs in the vasculature, neurons and glia resulting in pathological angiogenesis, vascular leakage and a loss in retinal function. All components of RAS have been identified in the retina and iris and it is likely that the local rather than systemic RAS is involved in ocular neovascularization. It was reported that the RAS components were upregulated in DR.

6.1 *ACE* gene

ACE, the main enzyme of RAS, is encoding by the *ACE* gene, composed of 26 exons, and span a total of 21 kb (Table 4). The genetic structure is made up of three ancestral regions, and two intragenic ancestral recombination breakpoints flank the gene region (Boright et al., 2005). Several polymorphisms have been reported in *ACE* gene. The two biallelic SNPs within and flanking the gene are in strong linkage disequilibrium with each other (Boright et al., 2005). The most extensively studied polymorphism was insertion/deletion of a *287-bp Alu* repeat in intron 16 (*rs179975; Ins/Del; I/D)* being considered a "reference" polymorphism (Hadjadj et al., 2007; Mooyaart et al., 2011). ACE activity is significantly connected with genetic variations at the *ACE* gene. The *rs179975* accounts for 44% of the interindividual variability of plasma ACE levels, and high ACE values were found among

subjects with *DD* genotype. Other report showed that about 24% of the variance in the ACE activity was attributed to other *ACE* polymorphisms *rs4343*, *rs495828* and *rs8176746* (Chung et al., 2010).

Some reports indicated in Caucasian T1DM patients that *ACE I/D* was not associated with the development of persistent microalbuminuria, or overt DN (Möllsten et al., 2008; Ringel et al., 1997; Tarnow et al., 2000). A protective role to the homozygosity for the insertion (*I/I*) of *ACE* gene in the DN development was attributed. With the increase of duration of diabetes it seems that the *ACE I/I* genotype is associated with longevity and survival in T1DM patients but not particularly in DN subjects (Boright et al., 2005). Other reports indicated that the *ACE D/D* genotype was more frequent in patients with DN and the presence of the *ACE D/D* or *I/D* genotypes was associated with a faster rate of the decline of the renal function, suggesting that the *ACE D* allele represents an increased risk for both the onset and the progression of DN (Costacou et al., 2006; Gumprecht et al., 2000; Ng et al., 2005; Nosikov, 2004).

From other *ACE* polymorphisms studied for the association with DN, it was reported that the *G7831A* (Nosikov, 2004), *rs4293* and *rs4309* (Currie et al., 2010) were not associate, while *rs1800764* and *rs9896208* (Boright et al., 2005) were associate with the disease. Regarding the *rs1800764* (*T/C*) variant, patients who carried the wild-type *T* allele were at lower risk for persistent microalbuminuria or severe DN, while heterozygous patients *(T/C)* had a higher risk for severe nephropathy, suggesting a genotype rather than an allele effect (Hadjadj et al., 2007). The reported haplotypic structure of *ACE* was considered to contain four polymorphisms *rs4311, rs4366, rs1244978* and *rs1800764 (*Hadjadj et al., 2007). Interestingly, the homozygosity for the common haplotype that carries the *ACE I* allele, as *TIC* haplotype, corresponding to the wild type alleles of *rs1800764*, *I/D* and *rs9896208*, respectively was associated with lower risk for development of severe DN. This finding provides a strong evidence that genetic variation at the *ACE* gene is associated with the development of DN (Boright et al., 2005). On the other hand it was reported that a haplotype containing the rare allele the *D* of *I/D* variant, *G* of *rs4366* and *G* of *rs12449782* was associated with a higher risk for DN (Hadjadj et al., 2007).

Diabetic nephropathy is rarely diagnosed using invasive kidney biopsies and generally in genetic studies DN patients were those who presented albuminuria. Conflicting results of the gene association with the disease might occur, in addition, from the fact that a substantial number of subjects were classified as having DN but actually have nondiabetic kidney disease instead. Certain investigators have proposed that DN cases should be required to have diabetic retinopathy as well. From 1994 up to 2006 there were numbers of reports analyzing T1DM patients with DN having in addition various proportion of DR (Ng et al., 2008). The relationship between *ACE* polymorphisms and DR was less studied. Most reports found no association of *ACE I/D* with the development of any form of DR in adult or younger Type 1 diabetic patients (Abhary et al., 2009a; Zhou & Yang, 2010). Other data showed that in patients with DR, the severity of DR was associated with *ACE I/D* polymorphism (Marre et al., 1997). While nearly all T1DM individuals can develop DR or DPN, only a fraction of the subjects develops DN. So, it is hard to determine whether any observed association between *ACE I/D* and DN or DR or combined DN/DR truly exists. Including DR in the identification of potential genetic factors for the microvascular disorders might help, considering that some patients manifest a joint retinal-renal phenotype (Ng et al., 2008).

Taken together, these data indicate that the *I/D* variant of *ACE* gene, considered a "reference" polymorphism, responsible, at least in part, for the interindividual variability of plasma ACE levels, is associated with the faster rate decline of renal function, particularly in patients with a less than 10 years of diabetes duration, and the *ACE D* allele represents an

increased risk for both the onset and the progression of DN. These findings were confirmed by multiple, independent studies. This potential genetic factor for DN development might be correlated also with DR, suggesting its involvement in the diabetic complex phenotype.

6.2 Other RAS genes

ACE2 has approximately 40% homology with ACE sharing 42% identity with the catalytic domain of somatic ACE, and promotes vasodilatation counterbalancing the ACE effect. The *ACE2* gene consists of 18 exons is stable and conserved, indicating, that the genetic effect is small, and it intertwines and functions in concert with many other genes, suggesting the presence of epistatic effects (Fröjdö et al., 2005; Zhou & Yang, 2010). Data of genes for other RAS components are presented in Table 4. From the variants (*rs714205; rs879922; rs1978124; rs2023802; rs2048684; rs2074192; rs2285666; rs4646188; rs5978731*) reported few studies implied genomic analyses of diabetes microvascular disorders. None of the studied polymorphisms were associated with DR (Currie et al., 2010; Fröjdö et al., 2005). An increased in ACE2 expression in early phases of diabetes in the absence of renal injury was reported (Giunti et al., 2006).

Angiotensinogen gene has more than 30 genetic polymorphisms as reported in different studies, *M24686*, *C1015T* (*T174M; rs4762; Thr/Met*), *T1198C (M235T*; *rs699*; *Met/Thr), A1237G (Tyr/Cys), A1204C* (*A-20C; rs5050), G1218A* (*G-6A; rs5051*). The most studied polymorphism in relation with diabetic microvascular disorders was *rs699* in exon 2 when a *T* to *C* base substitution at position 702 take place, with the consequent replacement of methionine 235 with threonine. A relationship between the *T* allele of *rs699* and increased plasma Ang II levels was reported only in male subjects and may account for no more than 5% of ANGT variability (Marre et al., 1997, Ruggenenti et al., 2008). Reports on the association of *AGT rs699* and the development of DN in adults with T1DM showed conflicting results; some finding indicated no association (Chowdhury et al., 1996; Currie et al., 2010; Hadjadj et al., 2001; Möllsten et al., 2008; Nosikov, 2004; Ringel et al., 1997; Tarnow et al., 2000), and others suggested that this variant contribute to the increased risk for chronic renal failure (Gumprecht et al., 2000). In young T1DM subjects, the *TT* genotype of *AGT rs699* had a fourfold increased risk for persistent microalbuminuria, suggesting that this variant is a strong predictor for early stage of DN (Gallego et al., 2008). One report analyzed *AGT T174M* in relation with DN, and the findings indicated no association with the disease (Nosikov, 2004). There were no reports indicated a significant relationship between *AGT rs699* and DR, but in patients with incipient diabetic renal failure the *T* allele of *AGT rs699* was associated with DR. In these patients interaction between the *D* allele of *ACE I/D* and the *T* allele of *AGT rs699* tended towards protection against DN (Van Ittersum et al., 2000). Oppose, other study indicated that the same interaction increases risk for DN in patients cu DR (Marre et al., 1997). All these data suggest that extensive studies has to be done in large number of T1DM patients with combined DN and DR vs only one microvascular disorder for the epistatic interactions between *ACE* and *AGT* polymorphisms and their relationship of the disease.

In *AGTR1* gene were identify a variety of polymorphisms *AF245699*, *A49954G* (*rs5183*; *A1878G*; *Pro/Pro*), *A50058C* (*rs5186*; *A1166C*), *T4955A* (*rs275651*), *T5052G* (*rs275652*), *C5245T* (*rs1492078*), *A1062G*, *T573C*, *G1517T (*Ruggenenti et al., 2008). Reports on the relationship of *AGTR1* polymorphisms with DN showed that the *AA* genotype of *rs5186* was independently associated with overt DN, being with a threefold increase in the risk for the disease

compared to *AC* and *CC* genotypes (Möllsten et al., 2008). The treatment with renoprotective antihypertensive (losartan) for slowing down the progression of diabetic glomerulopathy reduced significantly albuminuria, systolic and diastolic blood pressure in the *A* allele vs *C* allele carriers of *rs5186* polymorphism (Dragović et al., 2010). Oppose, the rs5186 of *AGTR1* was found not associated with DN in other reports (Gallego et al., 2008; Nosikov, 2004; Tarnow et al., 2000). There are no data on the influence of *AGTR1* gene polymorphism on the development of DR or DPN. For *AGTR2* gene have been identified few variants, *U20860* (*T3786C*; *rs5192*; *Ala/Ala*), *G1675A* (*rs1403543*), *G4297T* (*rs5193*), *A4303G* (*rs5194*) but there are no reports showing the involvement of one polymorphism with T1DM microvascular disorders.

Although the prognosis of patients with DN has improved, the decline in the GFR still varies among T1DM patients. The nongenetic risk factors (elevated blood pressure, albuminuria, and HbA_{1c}) for excessive loss of GFR, explain only approximately 30 to 50% of the decrease, and the epistatic interactions between *ACE*, *ACE2*, *AGT*, *AGTR1* or *AGTR2* polymorphisms in the RAS, a concept previously suggested (Jacobsen et al., 2003) might represent a risk factor for DN. It was reported that despite the non-significant effects of a single-gene on DN progression, a combined genetic variable including the potential "bad" alleles (*D* of *ACE I/D*, *M* of *AGT rs699*, and *A* of *AGTR1 rs5186*) represent a risk factor for the disease (Jacobsen et al., 2003). These data suggest that in some conditions a single gene variant may cause appreciable phenotypic changes only upon combination with other polymorphisms, having additional or synergistic effects on the same metabolic pathways (Ruggenenti et al., 2008). Oppose, other data showed that DN was not influenced by the epistatic interactions between the polymorphisms of the RAS genes. (Gallego et al., 2008; Tarnow et al., 2000)

7. Conclusion

All over the world, the incidence of type 1 diabetes continue to be much higher than in general population. Despite major progresses done in the recent years to identify candidate genes involved in the development of diabetic microvascular complications, there are still controversial results and insufficient knowledge in the literature, although a variety of genomic strategies were applied. While the degree of metabolic control remains the main risk factor for the development of diabetic chronic complications, the genetic risk factors, common for retinopathy, neuropathy, and renal disease, or specific for each of them, are important contributors to the disease severity. Discrepancies between reported data are due to differences in the genetic background between studied populations, small sample sizes, insufficient phenotype description, genotyping procedures, individual gene polymorphism assessment, few numbers of loci included in the studies, and requirement of interaction analysis between gene-gene variants. Genetic prediction and use of individual aetiological processes, as well as the translation of recent molecular knowledge into potential therapeutic agents will contribute selectively to the preventive and therapeutic interventions in this complex disease.

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9. References

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2

Early and Late Onset Type 1 Diabetes: One and the Same or Two Distinct Genetic Entities?

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1. Introduction

Type 1 diabetes is a complex autoimmune disease in which genetic and environmental factors add up to induce an autoimmune destruction of the insulin-producing pancreatic β cells. Although type 1 diabetes is popularly associated to an onset in infancy or adolescence, it can begin at any age. The reasons behind this temporal difference in the onset of the disease are probably a mixture of genetic and environmental factors, just as the induction of the disease itself. Despite the great progress that the study of the genetics of type 1 diabetes has experienced in the last years, the genetic factors that could modify the age at diagnosis of type 1 diabetes have not been analyzed so deeply. This knowledge would be interesting to discover new routes to delay the disease onset and preserve the β cell mass as long as possible. In this chapter, we will review the characteristics of adult-onset type 1 diabetes patients and afterwards we will focus on the studies in type 1 diabetes genetics and the reported associations of genetics and age at onset of the disease. Finally, we will present an analysis of ten genetic associations in a group of Spanish patients with early and late onset of type 1 diabetes.

2. Diagnostic criteria for diabetes and further classification of the disease

Type 1 diabetes is the most prevalent chronic disease in childhood and it is also the most frequent form of diabetes in subjects diagnosed before age 19 (Duncan, 2006). Adults can also suffer from type 1 diabetes, given that the prevalence rate does not vary greatly with age, but the diagnosis of the disease in adult age is complicated by the higher prevalence of type 2 diabetes, which is the most frequent form of diabetes in adulthood (American Diabetes Association [ADA], 2010). Classical diabetes classifications used to categorize patients by their age at diagnosis or insulin requirement. Thus, type 1 diabetes was termed juvenile diabetes or insulin-dependent diabetes mellitus (IDDM) and type 2 diabetes could be diabetes of the adult or non-insulin dependent diabetes mellitus (NIDDM). However, type 2 diabetes can begin as an insulin-dependent condition or at an early age, type 1 diabetes can begin at any age, and a certain form of adult onset autoimmune diabetes termed latent autoimmune diabetes of the adult (LADA) is non-insulin-dependent by

definition at the time of diagnosis, nevertheless it is an autoimmune form of diabetes. Therefore, cataloging the different forms of diabetes is not so simple and classifications based in age at diagnosis or insulin requirement are no longer employed. Hence, we will review the diagnostic criteria for diabetes and define what can be considered adult-onset type 1 diabetes.

According to the most recent classification of the American Diabetes Association (ADA, 2010), the diagnostic criteria for diabetes are 1) levels of glycosilated haemoglobin over 6.5%, 2) fasting plasma glucose levels over 126 mg/dl, defining fasting state as no caloric intake for at least eight hours, 3) plasma glucose over 200 mg/dl at two hours during an oral glucose tolerance test (OGTT) or 4) random plasma glucose over 200 mg/dl in a patient with classic symptoms of hyperglycemia (poliuria, polidypsia or glucosuria). Patients with type 1, type 2 diabetes or LADA must feet these criteria. Then, further classification of the patient should be considered.

2.1 Type 1 diabetes

Type 1 diabetes accounts for 5-10% of the total cases of diabetes and it is the 90% of the cases of diabetes diagnosed in children (ADA, 2010). The disease is an autoimmune condition characterized by the destruction of the pancreatic β cells by autoreactive T lymphocytes. Hyperglicemia manifests when 60-90% of the β cell mass has been lost. As a result of the autoimmune insult, antibodies against pancreatic islets are synthesized and can be detectable in serum (see table 1). These antibodies precede in several years the clinical symptoms. They are not pathogenic (Wong et al, 2010), but its detection helps in the classification of the patient as type 1 diabetes, especially when the disease is diagnosed in adulthood. Antibodies against pancreatic antigens are positive at diagnosis in 90% of type 1 diabetes patients. Obesity is quite uncommon in these patients, but not incompatible with the disease. Patients are frequently insulin-dependent since diagnosis and insulin-replacement therapy is ultimately necessary for survival. Also, C-peptide levels (a measure of β cell activity) are usually low or undetectable. When untreated, type 1 diabetes leads to diabetic ketoacidosis, a life-threatening condition derived from the use of fat deposits (ADA, 2010).

Adult-onset type 1 diabetes patients tend to have a softer disease onset, with a lower frequency of diabetic ketoacidosis and a slower loss of insulin secretion capacity (Hosszufalusi et al, 2003; Leslie et al, 2006). These characteristics lead to think that a slower autoimmune reaction is taking place in the patient with adult onset.

	Autoantibody	Antigen expression
IAA	Anti-insulin antibodies	Pancreas
GADA	Anti-glutamate decarboxilase antibodies	Pancreas/nervous system
IA2-A	Anti-insulinoma associated 2 antibodies	Pancreas
ICA	Anti-islet cell antibodies (several antigens)	Pancreas
SCL38A	Antibodies against the zinc channel ZnT8	Pancreas

Table 1. Autoantibodies against pancreatic antigens in type 1 diabetes.

2.2 Type 2 diabetes

Type 2 diabetes includes 90-95% of the total cases of diabetes and accounts for 80-85% of the cases diagnosed in adulthood (ADA, 2010). The disease is a result of a combination of peripheral insulin resistance and relative insulin deficiency that develops into hyperglycemia. The hyperglycemia in type 2 diabetes appears gradually, usually with absence of the classic symptoms (polyuria, polydipsia) and can go undetected for long time before diagnosis. The etiologic factors of type 2 diabetes are unknown and probably this trait, more than type 1 diabetes, is composed of several different diseases with the common clinical manifestation of hyperglycemia. However, there is no proof of an implication of an autoimmune response, and thus autoantibodies against pancreatic antigens are always negative (ADA, 2010). Patients are usually obese and non-insulin dependent, although insulin can become a necessary therapy for a good control of hyperglycemia in some cases. However, insulin treatment in type 2 diabetes is not required for survival. C-peptide levels can be lower than in healthy controls, reflecting the relative insulin deficiency, but they are higher than in type 1 diabetes patients and decrease more gradually with time (Hosszufalusi et al, 2003). Diabetic ketoacidosis is quite rare and tends to develop due to underlying conditions, such as an infection (ADA, 2010).

2.3 Latent autoimmune diabetes of the adult (LADA)

Early after the discovery of antibodies against pancreatic antigens in the serum of type 1 diabetic subjects, it was noticed by clinicians that 10% of patients first diagnosed with type 2 diabetes tested positive for type 1 diabetes antibodies (mainly GADA and ICA) (Palmer et al, 2005). Those patients slowly but relentlessly progressed to insulin-dependency and showed signs of pancreatic islet dysfunction such as a progressive decrease of C-peptide levels. These characteristics defined a new category in the diabetes spectra called the latent autoimmune diabetes of the adult, and abbreviated LADA. Diagnostic criteria for these patients are age at diagnosis over 30 years, presence of antibodies against pancreatic islets, with a higher frequency of single positivity and GADA or ICA antibodies than type 1 diabetes patients, and no requirement of insulin for at least six months since diagnosis (Palmer et al, 2005; Leslie et al, 2006). There seems to exist a similar but milder genetic background to that of type 1 diabetic patients (Hosszufalusi et al, 2003; Palmer et al, 2005), and some researchers think of LADA as a slower and less aggressive form of type 1 diabetes, to the point that this condition is usually termed type 1.5 diabetes. Debate exists over LADA being an entity of its own or just a less aggressive form of type 1 diabetes at an older age (Hosszufalusi et al, 2003; Palmer et al, 2005; Leslie et al, 2006; Steck & Eisenbarth, 2008). Anyway, this group of patients poses a very interesting subset for testing β cell preserving therapies in an autoimmune form of diabetes due to its slow progression to insulin-dependency.

2.4 The diagnosis of an adult-onset diabetic patient

When recruiting patients for genetic studies, a careful evaluation of adult-onset patients must be carried out to avoid misclassification. In most cases, the three more common forms of diabetes in adults can be distinguished with a test for antibodies against pancreatic antigens at diagnosis and the requirement for insulin therapy (see table 2).

	Adult-onset type 1 diabetes	Type 2 diabetes	LADA
Insulin requirement	Ultimately needed for survival	Useful for improved glycemic control in some cases	Not for the first 6 months after diagnosis. Patients eventually evolve to insulin-dependency
Corporal phenotype	Usually lean	Usually obese	Variable
Antibodies	2 or more positive	Negative	At least 1 positive High frequency of GADA+ and/or ICA+
C-peptide levels	Low or absent	Normal or slightly decreased	Low
Diabetic ketoacidosis	Present	Rare	Rare
T-cell response against pancreatic islet antigens	Positive	Negative	Positive
Type 1 diabetes HLA susceptibility	Present	Absent	Present
% of total adult diabetes	5-10%	80-85%	5-10%

Table 2. Summary of the characteristics of the three more common forms of diabetes in adulthood: adult-onset type 1 diabetes, type 2 diabetes and LADA (Leslie et al, 2006; ADA, 2010).

The subgroup of adult-onset type 1 diabetic patients is relatively easy to separate from the other two clinical manifestations of diabetes in adulthood: patients should be insulin-dependent since diagnosis, positive for at least two type 1 diabetes autoantibodies and usually of lean body type. However, many genetic studies in the last years have excluded the adult subset of type 1 diabetic patients as a precaution to avoid contamination with type 2 diabetic patients. This conservative measure has excluded a group of patients that could give us important information about the genetics of the disease: the existence of genes that modify the progression of the disease, together with unknown environmental factors, which would be the causatives of the fast immune β cell destruction in a child and the slower destruction in an adult patient.
From now on, we will focus on the genetics of type 1 diabetes and the study of the influence of genetics in age at disease onset.

3. Genetic studies in type 1 diabetes

The existence of a genetic basis that influences the development of type 1 diabetes is known since the first studies associated alleles and haplotypes in the Human Leukocyte Antigen (HLA) complex with type 1 diabetes risk in the late 70s (Nerup et al, 1974). Familial studies have been able to quantify the genetic basis of the disease in a range between 30-70% of the total contributing factors, being the remainder due to

environmental factors (Redondo et al, 2001a; Pociot et al, 2010). Half of this genetic contribution is due to alleles and haplotypic combinations in the HLA region (Erlich et al, 2008), which is the strongest genetic modifier of type 1 diabetes risk. The rest of the genetic load is composed of genes with smaller effects, some of which have been unveiled in the last thirty years through different approaches developed as the technology for DNA study evolved. These studies ranged from the association studies of candidate genes to the complex hypothesis-free genome-wide association studies. In this section, we will briefly review the methodology employed in these studies and its importance in the unraveling of the type 1 diabetes genetic component.

3.1 Association studies of candidate genes

The association studies are suited for the detection of variants with moderate or low effects on the disease, as long as the studied variants are relatively frequent in the population of study (minor allele frequency over 5%) (Pociot et al, 2010; Steck & Rewers, 2011). Association can be measured in a case-control design (study of the differences between a set of unrelated patients and healthy controls) or a family design (analysis of deviations in the theoretical 50% transmission of the variant from healthy parents to patients). Previous to the massive knowledge that the Human Genome Project provided to genetic studies in humans, association studies had to focus on the selection of candidate genes, which limited these studies to genes with known function and biased the selection by what was known (or believed) about the pathogenesis of the disease at the moment. Nevertheless, this approach discovered the five classical regions associated with type 1 diabetes and detailed in table 3.

First reported	Gene	Function
1970-1980 (Nerup et al, 1974)	HLA class II	Antigen presentation in antigen-presenting cells.
1984 (Bell et al, 1984)	*INS*	Expression levels in the thymus regulate the presence of insulin-reactive T cells.
1996 (Nistico et al, 1996)	*CTLA4*	Modulator of inactivation of the immune response. Constitutively expressed in regulatory T cells, a lymphocyte subset specialized in the suppression of autoimmunity.
2004 (Bottini et al, 2004)	*PTPN22*	Suppressor of signals through the TCR. Susceptibility variant is believed to favor the survival of auto-reactive T cells in the thymus.
2005 (Vella et al, 2005)	*IL2RA*	Alpha subunit of the high affinity IL2 receptor. Constitutively expressed in regulatory T cells, it is essential for the maintenance of this cell subset.

Table 3. Classical type 1 diabetes associated genes discovered through association studies of candidate genes. TCR: T cell receptor.

3.2 The genome wide association studies

The first genome-wide association study was published in 2007 (The Wellcome Trust Case-Control Consortium [WTCCC], 2007). Samples from seven diseases (among them type 1 and type 2 diabetes) were collected, recruiting 2000 cases of each disease and a common subset of 3000 healthy controls. This single study described four new regions strongly associated with type 1 diabetes, almost the same number of regions that the previous association studies had taken three decades to discover.

Genome-wide association studies were possible thanks to the great development in the knowledge of human genetics and in the techniques to study DNA, derived from initiatives such as the Human Genome Project. The design of a genome-wide study is based on the analysis of over 500.000 single nucleotide polymorphisms (SNPs) throughout the genome in each subject recruited. The study design is usually a case-control approach, but both case-control and family studies are commonly used to replicate the stronger associations in the recent genome-wide studies (Hakonarson et al, 2007; Barrett et al, 2009). Since their object of analysis is the whole genome, these kind of studies are hypothesis-free and are able to detect associations in regions and genes that a candidate gene study would have never considered, such as regions with genes of unknown function and genes in routes not classically considered to take part in the pathogenesis of the disease. Thus, from the four regions associated with type 1 diabetes and described in the WTCCC study (WTCCC, 2007), one (16p13) covered a gene of unknown function, and two (12q13 and 12q24) pointed to regions with several candidate genes.

Despite the advantage that poses the hypothesis-free design, the genome-wide association studies have a great disadvantage in return: the high number of polymorphisms studied implies an elevated number of statistical comparisons and an increased probability of obtaining false-positive associations. Therefore, these studies are subject to a strong statistical correction (WTCCC, 2007), and, depending on the number of markers analyzed, p values should be as low as 10^{-7} to be considered statistically significant (Todd et al, 2007). Moreover, the results obtained (especially those that are borderline significant) should be replicated in independent populations to assure that the result is not a false positive and it is not influenced by population variability (McCarthy et al, 2008). At this stage is where follow-up studies take place. Follow-up studies select associations from genome-wide studies for replication purposes, being the more interesting those that are borderline significant.

Genome-wide association studies and their follow-up have been successful in uncovering associations of high to moderate effect (odds ratio over 1.15) in variants with a minor allele frequency over 5%. Now, the remainder of the genetic component in type 1 diabetes is proposed to reside in rare variants with high effect and common variants with low effect on the disease (Pociot et al, 2010). Due to the stringent statistical correction required, genome-wide studies are not suitable for detecting these associations and new approaches will be necessary. Despite their limitations, in just five years genome-wide studies have revealed ten times more genetic regions than the older approaches did in thirty years, providing fifty genetic regions associated with type 1 diabetes. A brief summary of these regions can be consulted in table 4.

3.3 The problem with age at diagnosis

Although type 1 diabetes has a similar prevalence in all ages, the restriction to pediatric patients has been a popular criterion for recruitment of patients in studies on the genetics of the disease, as it is shown in table 5. Its purpose is to avoid the inclusion of misdiagnosed

Chromosome	Candidate gene	OR	Reference study	Published age-at-onset analysis
1p13.2	*PTPN22*	2.05	(Smyth et al. 2008)	Yes
1q31.2	*RGS1*	0.89	(Smyth et al. 2008)	
1q32.1	*IL20-IL10-IL19*	0.84	(Barrett et al. 2009)	
2q11.2	Several	--	(Barrett et al. 2009)	
2q24.2	*IFIH1*	0.86	(Smyth et al. 2008)	
2q32.2	*STAT4*	1.10	(Fung et al. 2009)	Yes
2q33.2	*CTLA4*	0.82	(Smyth et al. 2008)	
3p21.31	*CCR5*	0.85	(Smyth et al. 2008)	
4p15.2	*AC111003.1*	1.09	(Barrett et al. 2009)	
4q27	*IL2-IL21*	1.13	(Barrett et al. 2009)	
6p21	*HLA*	0.02-49.2	(Ounissi-Benkalha and Polychronakos 2008)	Yes
6q15	*BACH2*	1.13	(Cooper et al. 2008)	
6q22.32	*CENPW*	1.17	(Barrett et al. 2009)	
6q23.3	*TNFAIP3*	0.90	(Fung et al. 2009)	
6q25.3	*TAGAP*	0.92	(Smyth et al. 2008)	
7p15.2	Several	0.88	(Barrett et al. 2009)	
7p12.1	*COBL*	0.77	(Barrett et al. 2009)	
9p24.2	*GLIS3*	0.88	(Barrett et al. 2009)	
10p15.1	*IL2RA*	0.62	(Smyth et al. 2008)	Yes
10p15.1	*PRKCQ*	0.69	(Lowe et al. 2007)	
10q22.3	*ZMIZ1*	--	(Barrett et al. 2009)	
10q23.31	*RNLS*	0.75	(Barrett et al. 2009)	
11p15.5	*INS*	0.42	(Smyth et al. 2008)	Yes
12p13.31	*CLEC2D-CD69*	1.09	(Barrett et al. 2009)	
12q13.3	*CYP27B1*	1.22	(Bailey et al. 2007)	
12q13.2	*ERBB3*	1.31	(Barrett et al. 2009)	Yes
12q24.12	*SH2B3*	1.28	(Smyth et al. 2008)	Yes
13.32.3	*GRP183*	1.15	(Heinig et al. 2010)	
14q24.1	Several	0.86	(Barrett et al. 2009)	
14q32.2	Several	1.09	(Barrett et al. 2009)	
14q32.2	Several	0.90	(Wallace et al. 2009)	
15q14	*RASGRP1*	1.21	(Qu et al. 2009)	
15q25.1	*CTSH*	0.86	(Smyth et al. 2008)	
16p13.13	*CLEC16A*	0.81	(Smyth et al. 2008)	Yes
16p11.2	*IL27*	0.86	(Barrett et al. 2009)	
16q23.1	Several	1.28	(Barrett et al. 2009)	
17q12	Several	0.87	(Barrett et al. 2009)	
17q21.2	*SMARCE1*	0.95	(Barrett et al. 2009)	

Table 4. Summary of the 50 chromosomal regions currently associated with type 1 diabetes (continues in next page). The odds ratio in the table has been extracted from the reference study. Data come from the on-line database www.t1dbase.org, belonging to the Type 1 Diabetes Genetics Consortium (T1DGC). The reference study does not correspond with the published age-at-onset study. Genes *CLEC16A* and *SH2B3* were analyzed in Todd et al (2007) and where not associated with age at onset. The rest of the age-at-onset associations are reviewed in section 4.

Chromosome	Candidate gene	OR	Reference study	Published age-at-onset analysis
18p11.21	*PTPN2*	1.28	(Smyth et al. 2008)	Yes
18q22.2	*CD226*	1.16	(Smyth et al. 2008)	
19p13.2	*TYK2*	0.86	(Wallace et al. 2009)	
19q13.32	Several	0.86	(Barrett et al. 2009)	
19q13.4	*FUT2*	--	(Barrett et al. 2009)	
20p13	Several	0.90	(Barrett et al. 2009)	
21q22.3	*UBASH3A*	1.13	(Smyth et al. 2008)	
22q12.2	Several	1.10	(Barrett et al. 2009)	
22q12.3	*IL2RB*	--	(Barrett et al. 2009)	
22q13.1	*C1QTNF6*	1.11	(Cooper et al. 2008)	
Xp22.2	*TLR8*	0.84	(Barrett et al. 2009)	
Xq28	Several	1.16	(Barrett et al. 2009)	

Table 4 (continuation). Summary of the 50 chromosome regions currently associated with type 1 diabetes.

patients (type 2 diabetics or LADA patients) within adult-onset diabetic patients. However, enough criteria exist to discriminate type 1 diabetic patients from the remainder of diabetic adults, and the exclusion of adult type 1 diabetes patients limits the knowledge of the genetics of the disease only to its early onset. Adult-onset patients show signs of a slower immune reaction to β cells. The factors that cause a rapid destruction of β cells in a child but a slower degeneration in an adult-onset patient are unknown, nevertheless they are probably a mixture of genetic and environmental factors. Some hypotheses may explain the different speed in clinical manifestations: the genetic load of adult-onset diabetes could be composed of a lower number of associated genes than in the early-onset patients, or could be the same genes but with less effect in the adult disease, or maybe the adult-onset population has genes associated that are exclusive of adult-onset. Besides, the simple replication in adult-onset patients of associations found in pediatric type 1 diabetes is interesting to prove that, from a genetic perspective, adult-onset patients are as much type 1 diabetes as the pediatric-onset ones.

Four genome-wide studies and a series of follow-up have been published in type 1 diabetes in the last five years (table 5). Two included systematically some adult-onset patients in their populations; however most of these studies lacked an analysis of the influence of genetics in the age of diagnosis. The characteristics of the four genome-wide and some selected follow-up and major genetic studies, and the populations included in them, can be consulted in table 5.

The two populations that recruit late-onset patients deserve a more detailed commentary. The GoKinD (Genetics of kidneys in diabetes) population, included in Cooper et al in 2008, belongs to a United States project that aims at studying the genetics of kidney diseases in type 1 diabetes. Selection criteria for type 1 diabetes were age at diagnosis before 31 years, insulin therapy needed within the first year of diagnosis and not interrupted for any reason ever since. Patients had a minimum disease duration of 10 years. Analysis of the influence of genetics in age at diagnosis was not carried out in this genome-wide study.

Study	Year of publication	Population	Age limit for selection of participants	Study of genetics and age at diagnosis
(1) WTCCC	2007	2000 cases, British	Diagnosis before 17 years	No
(2) Todd et al	2007 Follow-up from WTCCC	4000 cases and 2997 British families	Diagnosis before 17 years	Yes
(3) Hakonarson et al	2007	563 cases and 1422 families from Britain, the US and Australia	Most diagnosed before 18 years	No
(4) Cooper et al	2008	GoKinD population 1785 US cases	Diagnosis before 31 years	No
(5) Smyth et al	2008 Major genetic study	8064 cases and 3064 families from US, Finland, Ireland, Norway and Romania	Diagnosis before 17 years	No
(6) Barrett et al	2009	T1DGC population 3983 cases and 2319 families from Britain, the US and Australia	Diagnosis before 35 years	No

Table 5. Genome-wide and major genetic studies performed in type 1 diabetes. Studies 2, 4 and 6 performed metanalysis with the WTCCC data. Also, the last genome-wide carried out by Barrett et al (study 6) performed a meta-analysis of the three larger genome-wide studies (1, 4 and 6) performed in type 1 diabetes.

The T1DGC (Type 1 Diabetes Genetics Consortium) is an initiative constituted in 2002 with the aim of providing resources to the research in type 1 diabetes. Since 2007, the consortium has published several studies on type 1 diabetes genetics (Erlich et al, 2008; Hakonarson et al, 2008; Howson et al, 2009; Qu et al, 2009) and, although the genome-wide study in which the T1DGC population was genotyped did not include an age-at-onset analysis, this group is lately including age-at-onset analyses in their publications and some of their studies have provided the first evidences of influence of genetics in age at onset of the post-genome wide era (Hakonarson et al, 2008). The selection criteria for adult patients are year at diagnosis under 35 years and uninterrupted insulin treatment for at least 6 months (Hakonarson et al, 2007). However, despite the wider limit in age at onset in this population, the majority of the patients included are pediatric, as reflected in the mean age at onset (around ten years) found in the published studies (Hakonarson et al, 2008; Howson et al, 2009).

4. Reported genetic associations of genes and age-at-diagnosis

The influence of genetics in age at onset of type 1 diabetes has been analyzed in some studies. However, initiatives to replicate these first studies or to establish a protocol to analyze the genetics or early and late onset are lacking, and therefore there is disparity in the definition and selection of late-onset type 1 diabetes patients, and in the statistical methods employed to analyze the associations. In this section, we will review some selected studies on the influence of genetics in age at diagnosis of type 1 diabetes.

- Familial studies: analysis in monozygotic twins with one member affected with type 1 diabetes have shown that the probability of developing the disease in the non-affected twin is considerably higher (38%) when the affected twin developed type 1 diabetes at an early age (under 24 years) than when the affected twin developed the disease after 25 years of age (6% risk for the non-affected twin) (Redondo et al, 2001b). This observation might suggest that early-onset type 1 diabetes has a stronger genetic component (responsible of the higher concordance rate) than the same disease with a late onset.
- HLA associations to age at onset: several studies (Redondo et al, 2001a; Leslie et al, 2006; Klinker et al, 2010) have pointed out to the higher risk and earlier onset of type 1 diabetes in patients that are heterozygote for the HLA class II risk haplotypes *DRB1*03* and *DRB1*04-DQB1*03:02*. On the other hand, the influence of HLA class I alleles in age at diagnosis has been thoroughly studied, and alleles *B*39* and *A*24* have been consistently associated to an earlier onset of the disease (Valdes et al, 2005; Nejentsev et al, 2007). The *B*39* allele, for example, precipitates the age of diagnosis in four years (Valdes et al, 2005).
- *IL12B*: the gene *IL12B* codes for the p40 subunit of the interleukin 12, also shared with interleukin 23. A 2004 study (Windsor et al, 2004) carried out in an Australian cohort including early and late onset patients found association of a polymorphism in position +1188 of the gene with late-onset of the disease (over 25 years). Neither associations on the *IL12B* gene with type 1 diabetes nor the described age-at-onset association have been replicated in recent studies.
- *CAPSL-IL7R*: this region was first associated with type 1 diabetes in a study of non-synonymous polymorphisms, finding a marker in the *CAPSL* gene that was highly associated with the disease (Smyth et al, 2006). Another polymorphism in the *IL7R* gene has been associated to type 1 diabetes and multiple sclerosis (Hafler et al, 2007; Todd et al, 2007). Our group undertook a replication study on both polymorphisms briefly after the discovery of the first signal (Santiago et al, 2008). We found association with type 1 diabetes in both polymorphisms and also described that both markers were associated with an earlier onset, an effect more noticeable in the *IL7R* polymorphism.
- Region 12q13 (*ERBB3* gene): in a replication study of borderline significant signals from a previous genome-wide (Hakonarson et al, 2007), the T1DGC found evidence of the association of three 12q13 polymorphisms with age at diagnosis (Hakonarson et al, 2008). The influence of this region on age at onset of type 1 diabetes has been subsequently analyzed in two independent studies (Awata et al, 2009; Wang et al, 2010) that, with different statistical methodology, did not replicate the effect seen in the first study. Finally, we have studied several signals in this region and found an age-at-diagnosis effect stronger than the previously described, with homozygotes for the susceptibility allele having an age at onset five years earlier than carriers of protective alleles (Espino-Paisan et al, 2011b).
- Region 2q32 (*STAT4* gene): a study in 2008 described an association of polymorphisms in the *STAT4* gene with type 1 diabetes patients with an onset earlier than 8 years (Lee et al, 2008). Among the polymorphisms studied was rs7574865, which we will include in our study on age at onset in section 5. This study was carried out in a pediatric Korean population, therefore population differences have to be taken in consideration, given that the genetics of Asian and Caucasian type 1 diabetes patients present some important differences (Ikegami et al, 2007).

- Insulin gene: the T1DGC group analyzed several classical type 1 diabetes genes (Howson et al, 2009) and found association of the susceptibility variant in the insulin gene to an onset of type 1 diabetes two years earlier than the protective allele. However, this effect was not replicated in one of the cohorts included in the study. We will analyze the same polymorphism in our study in section 5.
- *IL2RA*: a Finnish study analyzed several classical type 1 diabetes genes in a group with late-onset of the disease (Klinker et al, 2010). They found associations of the insulin, *PTPN22*, *IFIH1* and *CTLA4* genes with late-onset patients, and replicated the age-at-onset effect of the *DRB1*03-DRB1*04-DQB1*03:02* heterozygote. They also found that *IL2RA* was associated with an earlier disease onset. However, the T1DGC studied the *IL2RA* gene and did not find any effect in age at onset, although they did not include the stronger association in the gene that the Finnish study did analyze (Howson et al, 2009). Our group conducted a replication study in polymorphisms in the *IL2RA* gene and we found them associated to both early and late disease onset (Espino-Paisan et al, 2011c).
- *PTPN22*: a German group studied the C1858T polymorphism in the *PTPN22* gene and found that the susceptibility polymorphism was associated to an earlier onset of the disease in a group of pediatric-onset patients (Kordonouri et al, 2010). Patients with the susceptibility polymorphism had an onset of the disease two years earlier than homozygotes for the protective allele. However, this observation was not replicated in the T1DGC study (Howson et al, 2009). We will study this polymorphism in our group of pediatric and adult patients in section 5.
- *PTPN2*: our group studied the influence in age at disease onset of two polymorphisms in the *PTPN2* gene that had been previously associated with type 1 diabetes (Todd et al, 2007; WTCCC, 2007). We found that one of the studied polymorphisms was associated with an earlier disease onset, with carriers of the susceptibility allele having a disease onset almost three years earlier than homozygotes for the protective allele (Espino-Paisan et al, 2011a).

5. A practical study: Genetic analysis of a population with early and late-onset type 1 diabetes patients

We have selected ten chromosome regions (five classical genes and five genome-wide discoveries) previously studied in type 1 diabetes to test their association with pediatric and late-onset patients. We will briefly review their role in the pathogenesis of the disease and compare our results with the previous reported associations.

5.1 Population of study and methods

A total of 444 type 1 diabetes patients (47% female) were included in this study. All patients were recruited from the Madrid area (Spain), all where Caucasoid and diagnosed according to the criteria of the American Diabetes Association (ADA, 2010). Age at diagnosis was available for 415 patients and ranged from 1 to 65 years. Mean age at onset of the population was 18.6±11.1 years and median age at onset was 16 years. All patients were insulin-dependent since diagnosis and had been on uninterrupted insulin treatment for at least 6 months. Adult patients diagnosed over 35 years were included on the basis of positivity to autoantibodies, lean body type and insulin-dependency status. Also, a maximum of 888 ethnically matched controls (53.7% female) with no history of type 1 diabetes in first degree relatives were recruited.

Genes in the HLA complex were genotyped by two SSOP (Sequence Specific Oligonucleotide Probe) procedures: dot-blot hybridization and Luminex technology. The remaining genes were studied through genotyping of single nucleotide polymorphisms by TaqMan Assays in a 7900HT fast real-time PCR system (Applied Biosystems Foster City, CA, USA). The call rate for each SNP was 95%. A summary of these studied polymorphisms can be consulted in table 6.

Chromosome	Candidate gene	SNP	Assay reference	Control MAF
1p13	*PTPN22*	rs2476601	By design	T (0.06)
2q24	*IFIH1*	rs1990760	C___2780299_30	G (0.46)
2q32.3	*STAT4*	rs7574865	C__29882391_10	T (0.21)
2q33.2	*CTLA-4*	rs231775	C___2415786_20	T (0.29)
		rs3087243	C___3296043_10	G (0.48)
6q23.3	*TNFAIP3*	rs10499194	C___1575581_10	T (0.32)
9q33.2	*TRAF1*	rs2269059	C__15875924_10	A (0.07)
11p15.5	*INS*	rs689	C___1223317_10	A (0.28)
12p13.31	*CLEC2D*	rs11052552	C__32169467_10	G (0.49)
16p13.13	*CLEC16A*	rs2903692	C__15941578_10	A (0.42)

Table 6. Summary of the genotyped SNPs in each gene. MAF: minor allele frequency.

No statistically significant deviations from Hardy-Weinberg equilibrium were found in the control subset for each polymorphism. A case-control analysis was performed to asses association of the selected variants with type 1 diabetes. Differences were calculated through Chi-square and Fisher's exact tests when necessary. Analysis of age at onset was performed through a stratified and a continuous approach. For the stratified analysis, cases were classified in early-onset (age at diagnosis under 17 years) or late-onset (age at diagnosis over 16 years) and compared with Chi-square test or Fisher's exact test. Associations were estimated by the odds ratio (OR) with 95% confidence interval. All Chi-square and Fisher's exact test comparisons were calculated with Epi Info v.5 (CDC, Atlanta, USA). For the continuous analysis approach, ages at onset associated to each allele were compared with the non-parametric U Mann-Whitney test implemented in SPSS v.15.0 (Chicago, Illinois, USA).

5.2 Selected genes and results

5.2.1 Classical type 1 diabetes associations

Class II HLA alleles (chromosome 6p21): class II HLA binds extra-cellular antigens processed by antigen presenting cells and presents them to CD4+ helper T cells. The proposed mechanism in the pathogenesis of type 1 diabetes takes place at the negative selection process during lymphocyte thymic maturation (Redondo et al, 2001a; Ounissi-Benkalha and Polychronakos, 2008). Negative selection occurs when a T cell with an autoreactive T cell receptor (TCR) binds a HLA molecule loaded with an autoantigen. This union sends a strong activation signal through the TCR that is deleterious to the autoreactive T cell. Theoretically, susceptibility HLA alleles bind pancreatic antigens less efficiently, lowering the activation signal to non-deleterious levels and allowing the autoreactive T cell to escape from thymic selection.

The HLA associations detected in our group in the case-control analysis and the study on age at onset can be consulted in table 7. Due to the high number of alleles and haplotypic combinations in this region, and the low frequency of some of them, a stratified analysis would imply a marked loss of statistical power, so we choose to perform only the continuous analysis. We did not find evidence of an influence in age at diagnosis in any of the haplotypic combinations included, which means that our adult-onset patients have the same HLA contribution to type 1 diabetes than our pediatric patients. Of notice, there are two haplotypes with a marked difference in the mean age at diagnosis: the *DRB1*04-DQB1*03:02* homozygote (carriers have an onset three years earlier) and carriers of the protective haplotype *DRB1*15:01-DQB1*06:02* (carriers have an onset ten years later than non carriers). None of these comparisons are statistically significant, but it could be a problem of low statistical power since both genotypes are quite infrequent. A larger sample would be needed to elucidate the possible associations.

Many studies describe the *DRB1*03-DRB1*04* heterozygote as associated with earlier age at diagnosis of type 1 diabetes, and also as the haplotypic combination that confers a higher risk to the disease. In our population we do not see an age effect (p=0.9). Moreover, the *DRB1*03-DRB1*04* heterozygote does not confer the higher risk, but the *DRB1*04* homozygote, the *DRB1*03* or *DRB1*04* carrier, and the *DRB1*03* homozygote.

Insulin gene (chromosome 11p15): the insulin gene is also proposed to participate in the generation of autoreactive T cells in the thymus. The polymorphism associated with type 1 diabetes is a VNTR (variable number of tandem repeats) that locates upstream of the *INS* gene and modifies its expression in the thymus (Pugliese et al, 1997; Vafiadis et al, 1997). Alleles in this VNTR range from 26 to 210 repetitions of a consensus sequence and are usually classified in three groups: short class I alleles (26 to 64 repetitions), intermediate class II alleles (64 to 139 repetitions, infrequent in Caucasian and Asian populations) and large class III alleles (140 to 210 repetitions). Large alleles are associated with protection from type 1 diabetes and are related to a higher expression of insulin in the thymus (Vafiadis et al, 1997). This is thought to favor the negative selection of insulin-reactive T cells, a theory that would be consistent with the lower levels of insulin antibodies detected in patients that carry the large class III alleles (Hermann et al, 2005). We have selected a polymorphism (rs689) in linkage disequilibrium with the two main classes of alleles that is usually employed in genetic studies (Hermann et al, 2005; Todd et al, 2007; Smyth et al, 2008) as a proxy to the VNTR genotyping.

Genotype	Case-control analysis			
	Genotype frequency		p	OR
	T1D	Controls		
DR4-DQ8 homozygote	0.052	0.002	$6.0x10^{-8}$	33.59 (5.40-1386)
DR3-DQ2 carrier or DR4-DQ8 carrier	0.894	0.384	$2.1x10^{-61}$	13.24 (9.27-18.96)
DR3-DQ2 carrier	0.143	0.015	$3.1x10^{-16}$	11.19 (5.31-24.41)
DR3-DQ2 DR4-DQ8	0.249	0.031	$1.6x10^{-26}$	10.36 (6.11-17.75)
DR4-DQ8 – X (not DR3)	0.256	0.123	$3.7x10^{-8}$	2.43 (1.74-3.39)
DR3-DQ2 – X (not DR4)	0.369	0.221	$2.0x10^{-7}$	2.03 (1.53-2.69)
DR2-DQ6 – X	0.011	0.186	$4.5x10^{-19}$	0.05 (0.02-0.12)

Table 7. Case-control analysis of selected HLA haplotypes. Comparisons were calculated with Chi-square and Fisher's exact test when necessary. HLA haplotypes have been abbreviated: DR4-DQ8 (*DRB1*04-DQA1*03:01-DQB1*03:02*), DR3-DQ2 (*DRB1*03-DQA1*05:01-DQB1*02:01*), DR2-DQ6 (*DRB1*15:01-DQA1*01:02-DQB1*06:02*). T1D: type 1 diabetes.

Genotype	Continuous analysis		
	Mean age at onset		P
	Carrier	Non carrier	
DR4-DQ8 homozygote	15.9 (11.5)	18.6 (11.1)	0.2
DR3-DQ2 carrier Or DR4-DQ8 carrier	18.3 (11.1)	19.4 (10.2)	0.4
DR3-DQ2 carrier	17.5 (11.2)	18.6 (11.0)	0.2
DR3-DQ2 DR4-DQ8	18.0 (10.7)	18.6 (11.2)	0.9
DR4-DQ8 – X (not DR3)	18.0 (10.5)	18.9 (11.5)	0.7
DR3-DQ2 – X (not DR4)	18.8 (11.9)	18.4 (11.5)	0.9
DR2-DQ6 – X	28.0 (13.7)	18.5 (11.0)	0.2

Table 7 (continuation). Analysis of age at onset in selected HLA haplotypes. Comparisons were calculated with the U Mann-Whitney test. HLA haplotypes: DR4-DQ8 (*DRB1*04-DQA1*03:01-DQB1*03:02*), DR3-DQ2 (*DRB1*03-DQA1*05:01-DQB1*02:01*), DR2-DQ6 (*DRB1*15:01-DQA1*01:02-DQB1*06:02*). T1D: type 1 diabetes.

In our study, we replicate the association previously described in the *INS* gene and we do not find effects on age at onset in the stratified and continuous analysis. Results are provided in table 8.

Gene	CASE-CONTROL AND AGE-STRATIFIED ANALYSES				CONTINUOUS ANALYSIS		
	MAF		p	OR	Mean age at onset		P
					Major allele	Minor allele	
INS							
T1D-control	0.175	0.282	$8.3x10^{-9}$	0.54 (0.43-0.67)	19.3 (11.8)	18.0 (10.7)	0.4
Pediatric-adult T1D	0.162	0.177	0.6				
***CTLA4* rs231775**							
T1D-control	0.328	0.299	0.1	1.14 (0.96-1.37)	18.4 (10.9)	18.9 (11.4)	0.6
Pediatric-adult T1D	0.323	0.339	0.6				
***CTLA4* rs3087243**							
T1D-control	0.477	0.518	0.05	0.85 (0.72-1.00)	18.9 (11.0)	17.8 (10.8)	0.2
Pediatric-adult T1D	0.493	0.451	0.2				
PTPN22							
T1D-control	0.115	0.064	$7x10^{-6}$	1.91 (1.42-2.56)	18.4 (11.2)	19.0 (10.6)	0.4
Pediatric-adult T1D	0.113	0.127	0.5				
IFIH1							
T1D-control	0.373	0.409	0.1	0.86 (0.70-1.06)	17.2 (10.3)	17.2 (10.6)	0.9
Pediatric-adult T1D	0.373	0.394	0.6				

Table 8. Analysis of classical gene associations in type 1 diabetes. Minor allele frequency (MAF) is provided in case-control and age-stratified analyses, and comparisons were calculated with Chi-square and Fisher's exact test when necessary. In the continuous analysis, mean ages at onset associated to each allele and the p value from the U Mann-Whitney test are presented. *INS*: insulin gene. T1D: type 1 diabetes.

CTLA4 (chromosome 2q33): this gene encodes a negative regulator of lymphocytic activation. Its expression is induced in activated lymphocytes, but it is also constitutively expressed in regulatory T cells, a lymphocyte subpopulation specialized in the suppression of autoimmunity. Also, a soluble form of CTLA4 is secreted in the serum, and it is believed that this form contributes to the downregulation of activation in the immune system (Ueda et al, 2003). Several polymorphisms have been identified and associated with type 1 diabetes (Ueda et al, 2003; Qu et al, 2009). We have selected two functional polymorphisms: one aminoacidic change related to lower membrane expression of the protein (rs231775) and a polymorphism in the 3' end that is related to higher expression of soluble CTLA4 (rs3087243), which also is one of the strongest associations with type 1 diabetes in the gene (Ueda et al, 2003; Qu et al, 2009).

We replicate the association described in *CTLA4* rs3087243. Differences in *CTLA4* rs231775 do not reach statistical signification, but this could be due to low statistical power to detect the previously described association (Ueda et al, 2003). We do not find effects of any of the polymorphisms on age at onset. Results can be consulted in table 8.

PTPN22 (chromosome 1p13): this gene encodes a lymphoid-specific phosphatase called LYP, which is an important downregulator of T cell activation through the TCR. We selected the classical non-synonymous polymorphism C1858T that causes a substitution from arginine to tryptophan in the aminoacid 620 of the encoded protein (Bottini et al, 2004). The mutant form shows a higher phosphatase activity, and therefore it suppresses T cell activation more efficiently. Its role in type 1 diabetes is believed to be at the thymic selection process, where the mutant PTPN22 would lower the activation signal sent to the autoreactive T cell through its TCR, thus contributing to its survival (Bottini et al, 2006). It also has been proposed that the increased suppression of activation associated to the mutant form could affect negatively the activation of regulatory T cells (Bottini et al, 2006).

We replicate the association previously described in the *PTPN22* gene and we do not find effects on age at onset in the stratified and continuous analysis. Results can be consulted in table 8.

IFIH1/MDA5 (chromosome 2q24): certain viral infections such as that caused by Enterovirus are more prevalent in type 1 diabetes patients than in the healthy population, and it has been proposed that they could participate in the development or acceleration of the immune response against the β cell (Hober & Sauter 2010). The helicase IFIH1 recognizes viral double stranded RNA (dsRNA) and it is expressed in the cytoplasm of several cells, including β cells. In the presence of a viral infection, IFIH1 binds the dsRNA and induces the synthesis of pro-inflammatory cytokines. Functional experiments show that protection from type 1 diabetes is achieved through a lower performance of the sentinel role of IFIH1 that would end up in lower activation of the immune system in response to the viral infection (Colli et al, 2010).

We detect a lower frequency of the minor allele of *IFIH1* in type 1 diabetes patients respect to controls; however this difference is not statistically significant, probably due to low statistical power of our study. We do not find effects on age at diagnosis of type 1 diabetes in the stratified and continuous analysis. Results are provided in table 8.

5.2.2 Genome-wide associations

Region 2q32 (STAT4): the gene *STAT4* is an interesting candidate for type 1 diabetes. Member of a family of transcription factors, STAT4 activates the transcription of several genes including IFN-γ in response to interleukin-12 signaling. The pathway IL12-STAT4-IFNγ polarizes the immune response to a Th1 type, the kind of response that is thought to be responsible of the type 1 diabetes autoimmune reaction (Raz et al, 2005). We have selected a polymorphism that was first discovered associated with rheumatoid arthritis (Remmers et al, 2007). Our group studied this polymorphism in several autoimmune diseases and described its association with type 1 diabetes (Martinez et al, 2008), as it can be seen in table 9. Influence of this polymorphism in age at onset has been previously studied in a pediatric-onset Korean population, as we described in section 4. We performed a stratified and continuous analysis of age at onset and we did not find evidences of the influence of this polymorphism in age at onset of the disease. Results can be consulted in table 9.

Gene	CASE-CONTROL AND AGE-STRATIFIED ANALYSES				CONTINUOUS ANALYSIS		
	MAF		p	OR	Mean age at onset		P
					Major allele	Minor allele	
2q32 (*STAT4*)							
T1D-control	0.240	0.192	0.01	1.33 (1.05-1.68)	17.6 (10.6)	16.7 (9.4)	0.6
Pediatric-adult T1D	0.237	0.250	0.7	--			
6q23 (*TNFAIP3*)							
T1D-control	0.282	0.321	0.05	0.83 (0.69-1.01)	18.7 (11.1)	17.8 (10.6)	0.3
Pediatric-adult T1D	0.297	0.271	0.4	--			
9q33 (*TRAF1*)							
T1D-control	0.085	0.071	0.2	--	18.4 (11.1)	21.2 (10.5)	0.02
Pediatric-adult T1D	0.063	0.104	0.04	--			
12p13 (*CLEC2D*)							
T1D-control	0.462	0.504	0.06	0.85 (0.71-1.01)	17.6 (10.7)	18.5 (11.0)	0.4
Pediatric-adult T1D	0.471	0.460	0.8	--			
16p13 (*CLEC16A*)							
T1D-control	0.368	0.416	0.05	0.82 (0.66-1.01)	16.9 (9.7)	18.0 (11.5)	0.5
Pediatric-adult T1D	0.359	0.394	0.4	--			

Table 9. Analysis of selected gene associations from genome-wide studies in type 1 diabetes. Minor allele frequency (MAF) is provided in case-control and pediatric vs adult onset analyses, and comparisons are calculated with Chi-square and Fisher's exact test when necessary. In the continuous analysis, mean ages at onset associated to each allele and the p value from the U Mann-Whitney test are presented. T1D: type 1 diabetes.

Region 6q23 (TNFAIP3): two polymorphisms located in an intergenic space adjacent to the *TNFAIP3* gene have been associated to several autoimmune diseases, among them type 1 diabetes (Fung et al, 2009). We have selected the polymorphism that shows a stronger association with the disease. The gene *TNFAIP3* is expressed in β cells and serves as an anti-inflammatory mechanism by its downregulation of the NF-κB activation (Liuwantara et al, 2006); therefore, it poses an interesting candidate gene in the pathogenesis of type 1 diabetes. To our knowledge, this is the first time that this region is studied in relation to its influence in age at onset.

In our study, we replicate the association previously seen in type 1 diabetes and we do not find differences in age at onset either in the stratified or continuous analyses. Results are provided in table 9.

Region 9q33 (TRAF1): this region was first associated to rheumatoid arthritis (Kurreeman et al, 2007). Our group took part in a collaborative study that analyzed this region in several autoimmune diseases and found association with type 1 diabetes, among others

(Kurreeman et al, 2010). As an extension of the cited study, we selected and studied new polymorphisms in the *TRAF1* gene and analyzed their influence in age at onset of the disease. We present in table 9 the results obtained in one polymorphism that has not been previously studied in type 1 diabetes. Like *TNFAIP3*, the gene *TRAF1* is expressed in β cells and protects them against cytokine-mediated apoptosis in an inflammatory environment (Sarkar et al, 2009).

The polymorphism in *TRAF1* shows interesting data (table 9). We do not see statistical differences in the case-control analysis, but the age-stratified analysis shows an elevation of the minor allele frequency only in the adult-onset patients, that is statistically significant when compared to pediatric patients (p=0.04) and to controls (OR=1.52 [1.00-2.31]; p=0.04). On the other hand, the pediatric patients are similar to controls (p=0.6). The continuous analysis confirms the difference observed in the stratified analysis, showing an age at onset associated to the minor allele (mean 21.2) that is almost three years higher than the age at onset associated to the major allele (mean 18.4). Therefore, there seems to exist an association in this gene that is exclusive of our adult-onset type 1 diabetes patients.

Region 12p13 (CLEC2D): first associated with type 1 diabetes in the WTCCC study (WTCCC, 2007), it includes several genes. Polymorphisms associated with the disease (WTCCC, 2007; Barrett et al, 2009) have been identified in the surroundings of two genes with immunological function: *CLEC2D* and *CD69*. We will focus on the polymorphism near *CLEC2D* (coding the NK receptor LLT1), which was the strongest signal reported by the WTCCC (WTCCC, 2007) in this region. To date, the influence of region 12p13 on age at onset has not been studied.

In our study, we detect a trend towards association with type 1 diabetes of the studied polymorphism. We do not find differences in age at onset in the stratified and continuous analyses. Results are provided in table 9.

Region 16p13 (CLEC16A): this region was one of the most strongly associated with type 1 diabetes in the WTCCC (WTCCC, 2007) and was also discovered independently in a parallel study (Hakonarson et al, 2007). It covers a gene of unknown function termed *CLEC16A*, which is expressed in antigen presenting cells and NK cells, but little else is known about this gene or its possible role in the pathogenesis of the disease. Our group replicated the association detected in the WTCCC study (Martinez et al, 2010). Here we will analyze its influence in age at onset.

We replicate the association previously seen in type 1 diabetes. We do not find differences in age at onset in the stratified and continuous analyses. Results are provided in table 9.

5.3 Discussion

In this study we analyzed ten genetic regions, five of them classical type 1 diabetes genes and another five extracted from recent genome-wide studies. The analysis of age at onset (either age-stratified or considering age as a continuum) did not provide statistical differences in nine of the ten regions studied, therefore we propose that our adult-onset patients have the same genetic background in the studied genes that our pediatric-onset patients. Hence there is no reason to exclude these late-onset patients from genetic studies on type 1 diabetes. The results we observe in the classical type 1 diabetes susceptibility genes (table 8) are concordant with a recent Finnish study (Klinker et al, 2010) that analyzes these genes in late-onset patients and finds the same associations previously described in

pediatric-onset type 1 diabetes. Also, a recent study from the T1DGC (Howson et al, 2009) that replicated 19 genes, including *PTPN22*, *IFIH1* and *CTLA4*, studied age at onset in each one and did not find statistical differences, supporting the idea that the classical genetic associations to type 1 diabetes are shared between the early and late onset patients.

Interestingly, in our population we do not find differences in the HLA associations with early and late onset. It has been described before that the *DRB1*03-DRB1*04* heterozygote is the combination that confers a higher risk and it is associated with an earlier age at onset of type 1 diabetes (Redondo et al, 2001a; Leslie et al, 2006; Klinker et al, 2010). However, in our population the heterozygote is the fourth combination in risk conferred to the disease after the *DRB1*04-DQB1*03:02* homozygote, the *DRB1*03* or *DRB1*04-DQB1*03:02* carrier and the *DRB1*03* homozygote, and we do not see an effect on age at diagnosis of the heterozygote. This could be due to populational differences, quite important in the HLA complex. It is well known that not all the *DRB1*03* haplotypes confer the same susceptibility to the disease. An extended conserved haplotype marked by *B*18-DRB1*03-DQB1*02:01* is described to confer higher susceptibility among the *DRB1*03*-carrying haplotypes (Johansson et al, 2003; Urcelay et al, 2005). This haplotype is more frequent in the Mediterranean area and its frequency descends in Northern Europe. Therefore, it could be possible that the higher frequency of this high risk haplotype enhances the risk conferred by being a *DRB1*03* homozygote in a Mediterranean population such as the Spanish.

A recent study with the T1DGC family cohort (Howson et al, 2009) found a mild effect of the insulin gene in age at diagnosis of the disease, with the susceptibility allele conferring an onset two years earlier than the protective allele. We do not see an effect on age at diagnosis (continuous analysis, p=0.4). Moreover, in the aforementioned Finnish report, the authors also studied the *INS* gene and found association in late onset patients (Klinker et al, 2010). It is possible that our study lacks statistical power to detect a difference of two years in the age at diagnosis. However, the authors of the T1DGC study tried to replicate their findings in a case series of 900 patients and did not find the effect they saw in the family cohort, opening the door to the possibility that the described effect is a false positive.

Finally, we find that *TRAF1* is only associated to late-onset type 1 diabetes. Although our study is limited by low statistical power and would require replication in an independent late onset type 1 diabetes cohort, this is an interesting finding that would justify the study of the late-onset patients as a distinct set among the type 1 diabetes patients.

From the study of these ten genetic regions in our group composed of early and late onset patients, we propose that there are no major genetic differences between patients with an early and a late onset of type 1 diabetes.

6. Conclusions

Although the knowledge on the genetics of type 1 diabetes has experienced a great development in the last years, it has not provided many hints on the basis of the genetic components that could modify the age at onset of the disease. Several studies have approached the subject, but few of the reported associations have been properly replicated in independent populations. Also, the heterogeneity in the methodology of the published studies should be discussed: some studies select only paediatric patients. If the influence of a genetic region reaches a peak in the paediatric age and then decreases with

time, a study with paediatric and adult patients would better estimate the difference than a study with only paediatric patients in which the difference may be seen, but also may be smaller than it really is. The majority of the published studies analyze the age at diagnosis of type 1 diabetes as a continuous variable, but some studies adopt a stratified analysis that implies the fragmentation of the patients in two or more groups and the individual analysis of each subgroup. This strategy defines artificial groups with limits that do not have a biological justification, and makes it more likely to produce false results in underpowered studies. We recommend the age-stratified analysis as a screening method or as a confirmation analysis, but we consider the analysis of age as a continuous variable a more accurate method to detect differences, given that it only takes into consideration the genotype studied and the ages at onset of all the patients carrying this genotype. Therefore, we propose that age at diagnosis of type 1 diabetes should be studied in groups that include paediatric and adult onset patients, and that the statistical analysis should include at least one method that considers age at diagnosis as a continuum.

Despite the improvements that can be incorporated to age-at-onset analysis, what is known to date about the influence of the genetics on the early and late onset of type 1 diabetes allows us to formulate a tentative answer to the question that gives title to this chapter: are early and late onset type 1 diabetes the same or two distinct genetic entities? Our data, presented in section 5, and the previous studies reviewed in section 4 suggest that there are no major differences in the genetic component of paediatric and adult patients. Both share the risk conferred by the main type 1 diabetes risk modifiers such as the HLA, the insulin gene or *PTPN22*. However, two points have to be taken into consideration: first, minor differences can be found between the adult and the paediatric patient, such as the elevated prevalence of the higher risk HLA alleles in subjects with early onset. Second, the genetics of adult-onset type 1 diabetes patients has frequently been studied only in relation to genes that already showed association in paediatric patients. This approach precludes the possibility of discovering genes that could be associated exclusively to late-onset type 1 diabetes. The study of these two points is of great interest given that it could point to metabolic routes that take part in the acceleration of the disease and, therefore, genes in these routes would be excellent candidates for therapeutic strategies focused on the delay of the autoimmune β cell destruction.

In conclusion, we propose that type 1 diabetes, whether in its early or late-onset, is an autoimmune disease defined by a number of primary risk genes and a constellation of minor genetic modifiers that, together with environmental factors, define the pace of the autoimmune reaction that will determine the age at onset.

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3

Islet Endothelium: Role in Type 1 Diabetes and in Coxsackievirus Infections

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1. Introduction

The heterogeneity of microvascular endothelial cells derived from different organs, suggests that these cells have specialised functions at different anatomical sites. The microvasculature is, in fact, a key interface between blood and tissues and participates in numerous pathophysiological processes. Pancreatic islet microcirculation exhibits distinctive features, in an interdependent physical and functional relationship with β cells, from organogenesis to adult life. The islet microendothelium behaves as an active "gatekeeper" in the control of leukocyte recruitment into the islets during autoimmune insulitis in type 1 diabetes.

Furthermore, microvascular endothelial cells, forming the key lining between the vascular space and organ parenchyma, have been shown to influence organ and tissue specific susceptibility to viral infection, and to modulate the pathological expression of virus-induced diseases, which potentially includes type 1 diabetes. Endothelial cells expressing appropriate receptors would fail to act as effective barrier to infections, allowing viral particles to pass through, and replicate in, the vascular endothelium. Human Enteroviruses (EV), especially those of the Coxsackievirus B (CVB) group, are associated with a wide variety of clinical syndromes and have long been considered possible culprits of inflammatory conditions and immune-mediated pathological processes, such as chronic dilated cardiomyopathy, chronic myositis and type 1 diabetes mellitus (Rose et al., 1993; Luppi et al., 1998; Hyöty & Taylor, 2002). Several mechanisms, including molecular mimicry, bystander activation of autoreactive T cells, superantigenic activity of viral proteins, not mutually exclusive, have been proposed to explain the relationship between EV infections and induction of autoimmune diseases (Varela-Calvino & Peakman, 2003; Horwitz et al., 1998; Wucherpfennig, 2001). Evidences of a link between viral infections and initiation or acceleration of pancreatic islet autoimmunity have been under investigation for almost 30 years, and EVs, especially those of the Coxsackievirus B (CVB) group, are historically the prime suspects as important aetiological determinants in type 1 diabetes (Hyöty & Taylor, 2002; Varela-Calvino & Peakman, 2003). Endothelial cells derived from different organs show distinct susceptibility to CVB infections, and the behaviour against a viral challenge of endothelial cells in large vessels and microvessels may differ (Friedman et al. 1981; Huber et al., 1990; Conaldi et al. 1997; Zanone et al., 2003; Saijets et al., 2003).

2. Pancreatic islet microvasculature: Structure and specialised functions

It is widely accepted that remarkable heterogeneity of endothelial phenotype and function exists amongst different vascular beds (Kubota et al., 1988; Charo etal., 1984; Swerlick et al., 1991; Swerlick et al., 1992; Fujimoto & Singer, 1988; Lidington et al., 1999), in particular between cells derived from large versus small vessels, supporting the notion that tissue-specific vascular beds have specialised functions. These diversities include morphology, growth requirement in vitro (Kubota et al., 1988; Charo et al., 1984; Swerlick et al., 1991; Swerlick et al., 1992; Fujimoto & Singer, 1988; Lidington et al., 1999, Folkman et al., 1979) prostaglandin secretory profile (Charo etal., 1984), immunologic phenotype (Swerlick et al., 1992) and amounts and regulation of cell adhesion molecules (Swerlick et al., 1992; Fujimoto & Singer, 1988; Petzelbauer et al., 1993; Swerlick A.R., et al., 1992; Lee et al., 1992). At a functional level, differential and sequential expression of adhesion molecules mediates trafficking of leukocytes to specific lymphoid and non-lymphoid tissues.

Endothelium heterogeneity is the result of microenvironmental signals, in particular those induced by the family of vascular endothelial growth factor (VEGF) proteins (D'Amore & Ng, 2002). VEGF is a major stimulator of neovascularisation by inducing proliferation and migration of endothelial cells and tube formation. Pancreatic islets are one of the most vascularised organs, having a blood perfusion of about 10% of that of the whole pancreas, despite representing only 1% of the gland; this reflects high exchange demand with the endocrine cells and high metabolic supply (Figure 1). Deletion studies indicate that VEGF-A is responsible for this dense islet vascularisation, being more expressed in the endocrine than the exocrine pancreas (Lammert et al., 2003). Endothelial cells migrate to the source of VEGF-A, the neighboring β cells, proliferate and form blood vessels, organised in a network of sinusoidal capillaries reminiscent of those present in the renal glomerulus, with a five times higher density and ten times more fenestrations than in the exocrine tissue.

Islets receive blood from 1 to 3 arterioles and drain into collecting venules forming a network covering the islet surface, and an insulo-acinar portal system connects the islet capillaries with capillaries of the exocrine pancreas. The pattern of blood flow within the islet is still a matter of debate, with the β cell-rich islet core possibly perfused before the non-β cells in the periphery of the islet (Brunicardi et al., 1996).

Specific markers of islet microvasculature have been identified. These include the α-1 proteinase inhibitor (Api, α-1 antitrypsin), a major proteinase inhibitor with immuno-regulatory properties, and nephrin (Favaro et al., 2005), a highly specific barrier protein, known to be located in the renal glomerular ultrathin filter membrane "slit diaphragm" (Ruotsalainen et al., 1999; Tryggvason & Wartiovaara, 2001) (Figure 2). The nephrin expressed in islet microendothelial cells has functional characteristics that are highly reminiscent of the same protein expressed by podocytes in the renal glomeruli, in that in both cell types treatment with TNF-α acts on the cell cytoskeleton to induce a marked re-distribution of nephrin expression. Nephrin is a cell adhesion transmembrane protein of the immunoglobulin superfamily, which has a pivotal role in the regulation of renal glomerular selective permeability (Henderson & Moss, 1985; Bonner-Weir, 1993; Konstantinova & Lammert, 2004; Bonner-Weir & Orci, 1982). Islet endothelial expression of this protein is consistent with the ultrastructural features of these cells in the islets, which form a microvasculature that is characterized by a glomerulus-like network of fenestrated capillaries.

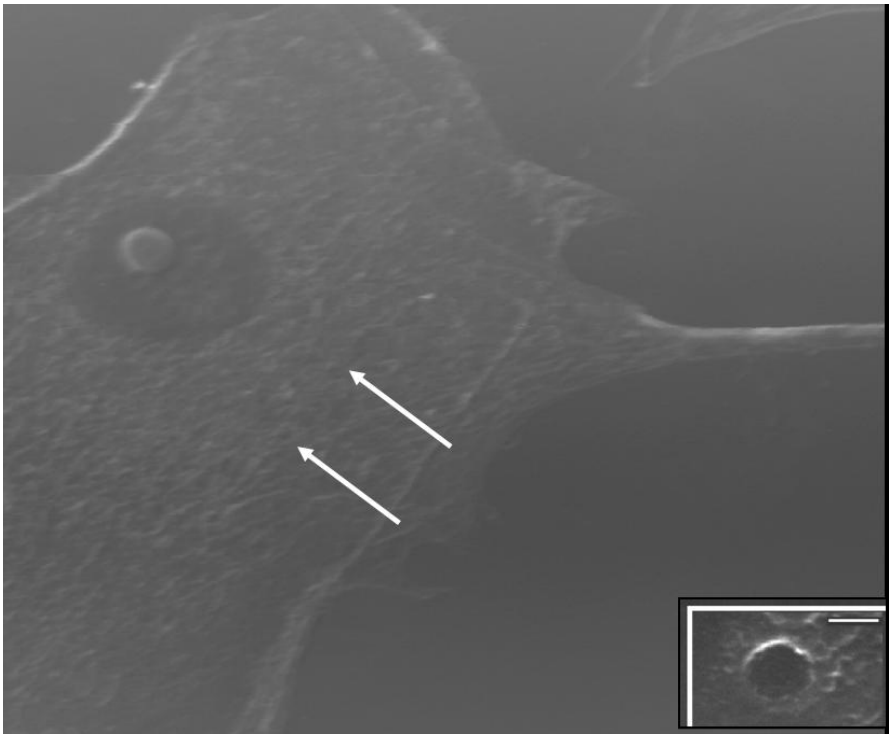

Fig. 1. Cultured islet endothelial cells.
Representative micrograph of scanning electron microscopy of primary islet MECs. The arrows show typical cellular fenestrations (original magnification 1500X). Inset: representative magnification of a cellular fenestra (original magnification 15,000X). Bar: 0.1 μm.

Nephrin appears to be more than just a structural component, as it is an adhesion and signalling molecule that can activate mitogen-activated protein kinase cascades, modulating a variety of cellular programs, including proliferation, differentiation and apoptosis (Karin et al., 1997; Flickinger & Olson, 1999). It has been shown that nephrin, once phosphorylated associates with PI3K and itself stimulates the Akt-dependent signaling pathway (Huber et al., 2003) that plays a pivotal role in preventing apoptosis in a variety of settings (Datta et al., 1999). In particular, Akt activation is crucial for the ability of factors such as insulin, IGF-I and VEGF to inhibit apoptosis in cultured endothelium (Jung et al., 2000). Recent data highlight the Akt role also in insulin-mediated glucose transport and pancreatic β cell mass and function (Bernal-Mizrachi et al., 2004; Elghazi et al., 2006).
Islet endothelium is crucially involved in fine-tuning blood glucose sensing and regulation (Lammert et al., 2003). Besides providing oxygen and nutrients to the endocrine cells, islet endothelium is in fact involved in the trans-endothelial rapid passage of secreted insulin into the circulation. In perfusion experiments with horseradish peroxidase it has been demonstrated that the endothelial fenestrae are sites through which proteins quickly permeate (Takahashi et al., 2002). Thus the islet fenestrae allow the fastest way for insulin to enter the circulation. Studies in mice with pancreatic VEGF-A deletion, showed that these

mice not only displayed loss of endothelial fenestrations and thicker endothelial cell body but also defective blood glucose levels on glucose tolerance test, pointing to a possible defect in the release of insulin (Lammert et al., 2003). A more recent study indicated that mice with β cell reduced VEGF-A expression show impaired glucose-stimulated insulin secretion, related to vascular alterations of the islets (Brissova et al., 2006).

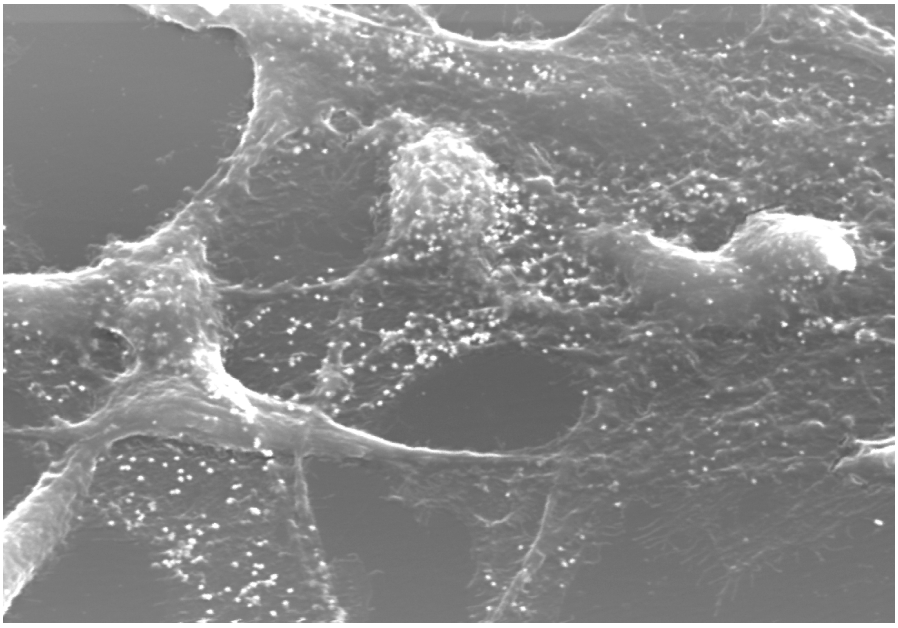

Fig. 2. Nephrin is expressed by islet endothelial cells.
Representative immunogold labelling micrograph of islet MECs stained with anti-nephrin Abs. By immunogold staining, nephrin appears distributed on the surface of islet MECs, without accumulation at cell-to-cell junctions (original magnification 1000X).

The microvasculature participates in sensing the environment of the islets and generates signals to affect adult islet endocrine function, being accepted that post-natal β cell mass is dynamic and can increase in function and mass to compensate for added demand (Bonner-Weir & Sharma, 2006). In an in vitro system, purified islet endothelial cells have been shown to stimulate β cell proliferation, through secretion of hepatocyte growth factor (HGF) (Suschek et al., 1994). VEGF-A and insulin are the islet-derived factor that induce HGF secretion. In vivo experiments, using pancreas of pregnant rats in which a high physiological proliferation of β cell occurs, showed prominent expression of HGF, coinciding with the peak of β cell proliferation.
Islet endothelium exhibits a unique phenotype also in the activities of the constitutive and cytokine-inducible endothelial nitric oxide (NO) synthases, forming the vasoactive mediator NO, since these enzymes are specifically regulated by the glucose level (Kolb & Kolb-Bachofen, 1992). This indicates an organ-specific control of NO production, whose role in islet cytotoxicity is well established (Kroncke et al., 1993; Steiner et al., 1997; Southern et al., 1990; Schmidt et al., 1992). The role of NO in the physiology of insulin release instead

remains controversial (Welsh & Sandler, 1992; Corbett et al., 1993; Henningsson et al., 2002; von Andrian & Mackay CR, 2000; Ostermann et al., 2002).
Immunohistochemical studies have shown that the expression of Platelet-activating factor (PAF) receptor (PAF-r) within the islet, is restricted to endothelial cells, providing potential target for therapeutic intervention (Biancone et al., 2006). PAF, is a phospholipid with diverse physiological effects that mediates a host of biochemical activities, including angiogenesis and inflammation. Islet endothelial cells have also been shown to rapidly produce PAF under stimulation with trombin, and PAF accelerated angiogenesis (Mattsson et al., 2006). These data suggest that intra-islet production of PAF, induced by inflammatory mediators, may contribute to the neovascularisation of transplanted islets.
Lastly, islet endothelial cells express genes encoding for a number of other factors involved in angiogenesis, including potent pro-angiogenic factors, such as VEGF, and angiostatic factors, such as endostatin and pigment epithelial-derived factor (Lammert et al., 2001).

2.1 Endothelial signalling during development and interplay between endothelial and β cells

Elegant experiments on early pancreatic development demonstrated that blood vessel endothelium in the dorsal aorta provides inductive signals for the differentiation of the primitive endoderm into islet cells (Lammert et al., 2001; Yoshitomi & Zaret, 2004). *In vivo* embryonic manipulation of frog embryos to block the formation of the dorsal aorta endothelium, leads to failure of pancreatic gene and insulin expression. To assay blood vessel-pancreas interactions later in development, VEGF-A was overexpressed in transgenic mice using the pancreatic promoter, *Pdx1*; this leads to hypervascularisation of the pancreas and hyperplasia of the pancreatic islets (threefold increase in islet area), at the expense of acinar cell types. Further, coculture experiments with endoderm and dorsal aortic endothelium from early mouse embryos, result in pancreatic gene *Pdx1* induction and insulin expression, indicating that endothelial signals are sufficient for the pancreatic organogenesis program. A successive study on pancreatic organogenesis, has shown that aortal endothelial cells induce in the dorsal pancreatic endoderm the crucial pancreatic transcription factor Ptf1a, that has been shown to be necessary for the development of endocrine and duct cell lineages.
A two sep-model for islet development has been proposed (Konstantinova & Lammert, 2004): the first step involves signals from the endothelium to the pancreatic epithelium, the second involves signals in the opposite direction, with islets expressing VEGF-A at later stages of their development to attract capillaries. As for the molecular basis for such signals, recent studies indicate that β cells, by using VEGF-A attract endothelial cells, which form capillaries with a vascular basement membrane next to the β cells. In turn, laminins, amongst other vascular basement membrane proteins, regulate insulin gene expression and β cell proliferation; these effects require β_1 integrin on β cells (Nikolova et al., 2006).
Studies on β cell proliferation in humans are limited, but there is evidence that this process occurs at relatively high levels in the first 2 years of life, declining thereafter, with the possibility, at least in animals, of re-induction under conditions of insulin-resistance, such as pregnancy or obesity (Meier et al., 2008; Cnop et al., 2010). This suggests that β cell may retain an intrinsic capacity to replicate, and an increase of the islet vasculature has been observed in association with conditions of expanded islet mass (Mizuno et al., 1999; Like

1970; Predescu et al., 1998). Islet endothelium-derived hepatocyte growth factor (HGF) is one of the factors potentially involved in the stimulation of β cell proliferation (Suschek et al., 1994).
Also collagen IV and other basement membrane proteins, laminins, could potentiate insulin secretion, promote insulin gene expression and proliferation in β cells, via interaction with integrin $\alpha_1\beta_1$ on β cell (Treutelaar et al., 2003; Kroncke et al., 1991).
These studies confirm the existence of an endothelial-endocrine axis within adult pancreatic islets.

3. Islet endothelium and type 1 diabetes

Islet endothelium forms the barrier across which autoreactive T cells transmigrate during the development of islet inflammation in autoimmune diabetes. Transendothelial migration and recruitment of autoreactive T cells into the pancreatic islets is a critical event during the development of chronic insulitis in type 1 diabetes. Transmigration is a complex, multistep process involving first selectins and their counter ligands that induce rolling of cells along the luminal surface of endothelial cells, followed by firm adhesion between cells and endothelium, and diapedesis (von Andrian & Mackay, 2000).
Several human and murine studies indicate that during autoimmune insulitis, the endothelial cells surrounding the inflamed islets adopt an activated phenotype, upregulate a variety of adhesion molecules, and are likely to be involved in regulating mononuclear cell accumulation (transmigration and homing) in the islets (Hanafusa et al., 1990; Hanninen et al., 1992; Hanninen et al., 1993; Itoh et al., 1993; Somoza et al.,1994). Activation of the islet endothelium may either initiate or enhance subsequent leukocyte infiltration of the islets. The islet endothelium is able to hyperexpress adhesion molecules, to secrete numerous cytochines and chemokines, and to hyperexpress class I and class II HLA molecules (Itoh et al., 1993; Somoza et al.,1994; Alejandro et al., 1982). Endothelial cells participate also in presentation of cognate antigens to T cells, which has potent effects on their migration in vitro and in vivo (Epperson & Pober, 1994; Marelli-Berg et al., 1999; Marelli-Berg et al., 2004; Pober et al., 2001) .
In particular, in humans, immunohistological studies of islets obtained near to the time of type 1 diabetes diagnosis, show abundant adhesion molecule expression on vessel and immune cells. In particular, bioptic studies showed that infiltrating mononuclear cells consisted of $CD4^+$ T, predominant $CD8^+$ T and B lymphocytes and macrophages, accompanied by increased expression class I and class II HLA antigens in endothelial cells (Itoh et al.,1993; Greening et al., 2003; Lozanoska-Ochser & Peakman, 2005). Pancreatic islet endothelial MHC class I hyperexpression has been observed also in NOD mice and the bio-breeding rat model of autoimmune diabetes (Kay et al., 1991; Ono et al., 1988), and represents a mechanism through which tissue-specific migration of T cells is refined and promoted.
In support to this, human islet endothelial cells have been shown *in vitro* to be capable of internalizing, processing and presenting to autoreactive CD4 T cell clones, disease-relevant epitopes of the islet autoantigen GAD65 (Greening et al., 2003; Di Lorenzo et al., 2007). This resulted in markedly enhanced transmigration.
Islet endothelial cells have also been shown to possess the necessary repertoire of the costimulatory molecules for adequate T cell activation. In vitro studies on the molecular

interactions between generated human islet endothelial cells and autoreactive T cells, indicate that islet endothelial cells constitutively express the CD86 (B7-2) and ICOS-L, but not CD80 (B7-1) and CD40 costimulatory molecules. Such co-stimulatory molecules are capable of functionally co-stimulating $CD4^+$ T cell activation, and to help activated memory ($CD45R0^+$) CD4 T cells to migrate across the endothelial barrier (Lozanoska et al., 2008). These studies provide strong indication that islet endothelium actively participates in the recruitment of recently activated lymph node migrant autoreactive T cells. Blockade of the costimulation may represent a mode of *in vivo* action of intervention therapies that interfere with costimulation, such as CTLA-4 Ig (abatacept). Furthermore, analysis of the immunophenotype of endothelial cells, focusing on endothelial MHC class I molecule expression, in a range of different tissues and mouse strain, including the NOD mice, shows that MHC levels have a profound effect on activation, adhesion and transmigration of pathogenic, islet autoreactive CD8 T cells (Lozanoska-Ochser & Peakman, 2009). These finding have a direct relevance to the pathogenesis of autoimmune diabetes in the MOD mouse, and are in concert with those with Savinov *et al*. (Savinov et al., 2001) who demonstrated that homing of a diabetogenic insulin-specific CD8+ T cell clone was severely impaired when clone cells were infused in IFN-γ knock-out mice, despite normal adhesion to the microvasculature. More recently, the same authors showed that islet-specific homing of the same diabetogenic clone depends in part upon recognition of the cognate MHC/peptide complexes presented by pancreatic islet endothelial cells, which are presumed to acquire insulin from adjacent β cells (Savinov et al., 2003).

Based on these observations, it is proposed the model in which, during islet inflammation due to as-yet non-defined environmental insult (possibly a viral infection), cytokines and other inflammatory mediators, such as IFN-γ, are released and elicit activation of vascular endothelium. Endothelial activation leads to increased adhesion and extravasation of leukocytes. Further, insulin, to high level of which endothelial cells are chronically exposed, and islet antigens released by damaged β cells, may be taken up by activated endothelial cells, processed and presented to autoreactive T cells.

Furthermore, sustained and intermitted hyperglycemia has been shown to affect endothelial cellular survival and proliferation, including islet microendothelium (Favaro et al., 2008). Several metabolic mechanisms are involved, including oxidative stress, increased intracellular Ca^{++}, mitochondrial dysfunction, changes in intracellular fatty-acid metabolism, impaired tyrosine phosphorylation and activation of PI3K/Akt and ERK1/2 pathways and reduced intracellular cAMP and its target, the cAMP-dependent PKA pathways (Datta et al., 1999; Favaro et al., 2010). These multifunctional pathways transmit signals that result in prevention of apoptosis or induction of cell cycle progression, depending on the cell type and can cross-regulate one another (Stork & Schmitt, 2002). Akt signaling cascade has also a role in insulin-mediated glucose transport and pancreatic β-cell mass and function (Bernal-Mizrachi et al., 2004; Elghazi et al., 2006). Pro-survival Bcl-2 protein, which stabilizes the mitochondrial membrane and prevents the release of cytocrome c from the mitochondria and the activation of caspases (Choy et al., 2001), is also found to be down-regulated by high glucose in human islet microendothelial cells. In contrast, the pro-apoptotic member Bax, which antagonizes Bcl-2, is up-regulated (Favaro et al., 2010). It is noteworthy that over-expression of Bcl-2 in endothelial cells has been described to decrease T cell cytotoxicity, suggesting that this protein may also protect endothelial cells from apoptosis resulting from an immunological insult (Zheng et al., 2000).

Due to the established interdependent physical and functional relationship between islet endothelium and β cells, from pancreatic organogenesis to adult life (Zanone et al., 2008), and the notion that post-natal β -cell mass is dynamic and can increase in function and mass for added demand by replication or neogenesis, possibly through endothelial inductive signals (Nikolova et al., 2006; Johansson et al., 2006; Bonner-Weir & Sharma, 2002; Dor et al., 2004), these high glucose-induced changes in islet endothelium carry relevant consequences on β cells. In fact, production of the vasoactive mediator NO (Meier, 2008; Favaro et al., 2008) to upregulate CD40L expression in human islet microendetelial cells *in vitro* (Favaro et al., 2010). Functional CD40L is expressed on vascular endothelium (Mach et al., 1997) and contributes to B cell activation, isotype switching, costimulation in T cell mediated immunity, activation of extravasating monocytes (Yang & Wilson, 1996; Wagner et al., 2004), with an impact in atherosclerosis and in chronic inflammatory and autoimmune diseases. Blockers of the CD40L have been strikingly effective in animal models of autoimmune diseases, such as systemic lupus erythematosus and type 1 diabetes (Homann et al., 2002). Therefore, high glucose-induced overexpression of CD40L on islet endothelial cells might accelerate the targeting and loss of the remaining β-cell capacity during ongoing autoimmune insulitis.
In fact, production of the vasoactive mediator NO by islet endothelium (Meier, 2008; Favaro et al., 2008) is increased in hyperglycaemic conditions and has an established direct cytotoxicity on islets and potentially impairs insulin release (Corbett JA et al., 1993). Islet microendothelial cells also are source of the proinflammatory cytokine IL-1β under hyperglycaemic conditions, independently of any viral or immune-mediated process. IL-1β impairs insulin release in human islet, induces Fas expression enabling Fas-mediated apoptosis and it is implicated as a mediator of glucotoxicity (Maedler K, et al. 2002). The high glucose condition is also reported to upregulate CD40L expression in human islet microendothelial cells in vitro (Favaro E et al., 2010).

4. Enteroviruses and type 1 diabetes

Viral infection has been long implicated in the development of type 1 diabetes and evidences of a link between viral infections and initiation or acceleration of pancreatic islet autoimmunity have been under investigation for more than 30 years. Rubella virus (Karvonen et al., 1993), mumps virus (Hyoty et al., 1988), cytomegalovirus (Ward et a., 1979), rotavirus (Honeyman et al., 2000) and enteroviruses (EV) (Lonnrot et al., 2000; Stene et al., 2010) have all been suggested as environmental factors contributing to type 1 diabetes. EV, especially those of the Coxsackievirus B (CVB) group (Hyöty et al., 1988; Varela-Calvino & Peakman , 2003; Green et al., 2004), are historically the prime suspects as important aetiological determinants and seroepidemiological, histopathological, animal studies, and *in vitro* experiments have provided the strongest overall evidence for these viruses. The EV genus of the Picornaviridae family is a large group of human pathogens traditionally divided into polioviruses, coxsackieviruses, echoviruses and the new EV, and each group contains a range of serotypes (King et al., 2000; Roivainen, 2006). Human EV are the most common cause of viral infection in humans, are associated with a wide variety of clinical syndromes and have long been considered possible culprits of inflammatory conditions and immune-mediated pathological processes, such as chronic myocarditis, dilated cardiomyopathy and chronic myositis (Tam, 2006; Luppi et al., 2000). In the cardiac context, injury is caused by a direct cytopathic effect of the virus, an immune response to viral infection or autoimmunity triggered by the viral infection (Huber, 2006).

Several mechanisms, including molecular mimicry, bystander activation of autoreactive T cells, superantigenic activity of viral proteins, viral infection and persistence, not mutually exclusive, have been proposed to explain the relationship between EV infections and induction of autoimmune diseases (extensively reviewed in Varela-Calvino & Peakman, 2003; Ercolini & Miller, 2009).

As for a role in type 1 diabetes, results have been somewhat conflicting and not conclusive (von Herrath, 2009; Tauriainen et al., 2010). Autoantibodies to islet autoantigens are detected years prior to diagnosis of type 1 diabetes and prospective studies evaluating whether EV could predict islet autoimmunity have yielded conflicting results, with positive associations in the Finnish studies (Lönnrot et al., 2000; Salminen et al., 2003; Sadeharju et al., 2003), and no association in other reports (Graves et al., 2003; Füchtenbusch et al., 2001). Discrepancies could be related to the fact that in most studies the determination of EV infection was carried out indirectly through the determination of IgM and IgG anti-EV antibodies, while studies using multiple approaches to identify EV infection (serology, RT-PCR, faeces analysis) appear more likely to report an association with type 1 diabetes or islet autoimmunity. A higher frequency of EV RNA has been consistently shown in the serum of patients with diabetes compared to healthy control subjects, demonstrating a recent or a persistent infection (Lönnrot, M., Salminen, K., et al., 2000; Lönnrot, M., Korpela, K., et al., 2000), and in some of the cases the detection of EV RNA preceded the synthesis of islet cell autoantibodies. In most studies viruses of the CVB group, usually CVB3 and CVB4 were identified (Clements et al., 1995; Andréoletti et al., 1997; Chehadeh et al., 2000), in agreement with serological studies.

As for T cell responses to EV, studies are inconclusive. However, it has been shown that CD4 T cells from newly diagnosed patients up-regulate CD69 early activation marker after exposure to CVB4-infected lysates (Varela-Calvino et al., 2001) and produce more IFN-γ a pro-inflammatory cytokine generated by effector memory CD4 T cells, but show less T cell proliferation (Varela-Calvino & Peakman, 2003). Proliferation is dependent upon IL-2 secretion associated with central memory T cells. This implies that anti-CVB4 effector cells are mobilized from the central memory pool, which may be depleted. Response to CVB4 antigens at diabetes diagnosis appears thus to be active, indicating recent or prolonged exposure.

Results from animal models indicate that viral infections *per se* usually cannot initiate the autoimmune disease process leading to diabetes, but may accelerate an already ongoing disease process. Studies in various NOD mice strains show that EV infections may accelerate the progression to diabetes only if they occur after autoreactive T cells have been accumulated in the islets (Hiltunen et al.,1997; Lönnrot et al., 1998; Lönnrot et al., 2000; Otonkoski et al., 2000). CVB infection appears to accelerate the development of the disease via bystander activation of autoreactive T cells, due to inflammation of the pancreas, tissue damage, release of sequestered autoantigens in concert with production of pro-inflammatory cytokines, all leading to activation of autoreactive T cells, but apparently only when a certain threshold of these autoreactive T cells have already accumulated in the pancreas. Timing of a CVB infection, rather than its simple presence or absence, may thus have etiological implications for the development of type 1 diabetes.

A recent report evaluating whether such a general model of disease progression rather than initiation by EV applies to human type 1 diabetes (Stene et al., 2010), suggests that progression from islet autoimmunity to type 1 diabetes in high-risk individuals may increase after an EV infection characterised by the presence of viral RNA in blood. Indeed, most EV are avid triggers of production of pro-inflammatory cytokines by human

leukocytes (Vreugdenhil et al., 2000), notably type I interferons, and it is noteworthy that increased levels of IFN-γ have been detected in the blood of newly diagnosed patients (Chehadeh et al., 2000), and EV RNA was detected in half of the IFN-α positive patients. These data are consistent with a recent EV infection.

4.1 Enteroviruses and pancreatic islets

Major determinants of the different clinicopathological manifestations of EV infections, ranging from silent infections to autoimmune diseases, are represented by the viral variants, the nature of the infection, acute, chronic or re-infection, and the distinct tissue tropism of the viral strain, modulated by the local expression of appropriate cellular receptors and coreceptors. The first step in viral infection is the attachment of the virus to its receptor, a cell surface molecule which viruses have adapted to use for their entry into the cells. These include the Coxsackie-Adenovirus receptor (CAR), integrin VLA-2, $\alpha_v\beta_3$, $\alpha_v\beta_5$, ICAM-1 and decay-accelerating factor (DAF) (Bergelson et al., 1997; Noutsias et al., 2001; Shafren, 1998). In cultured cells, CVB have been found to interact with at least three receptors. CAR is a 46kD adhesion molecule and all tested clinical and laboratory isolates bind to this receptor (Bergelson, 2002; Kallewaard et al., 2009). A large subset of CVB isolates also binds to DAF, a complement regulatory protein (Coyne & Bergelson, 2006) which appears to act as a receptor for cell attachment (Shafren et al., 1995), and some CVB3 isolates have been shown to use a third receptor, heparan sulfate, to infect CAR-deficient cells *in vitro* (Zautner et al., 2003).

These receptor molecules do not simply bind viruses, but may activate a series of events influencing the organ-specific outcome of disease (Ito et al., 2000; Selinka et al., 2004). CAR expression, for instance, is positively related to the extent of inflammation in the cardiac myosin-induced myocarditis model (Ito et al., 2000), and knockout of MyD88, an adaptor involved in toll-like receptor signaling, causes reduced cardiac expression of CAR and pro-inflammatory cytokines (Fuse et al., 2005) or TGF-β reduced CAR levels inhibit CVB3 infection of cardiac myocytes (Shi et al., 2010). CAR appears as the major receptor mediating CVB infection also in the pancreas *in vitro* and *in vivo*, since tissue-specific CAR gene deletion generated a 1000-fold reduction in virus titres within the pancreas during infection, and a significant reduction in virus-induced pancreatic tissue damage and inflammation (Kallewaard et al., 2009).

While acute infection in the pancreas has been clearly detected among the cells of the exocrine tissue, β cell infection by EV has been extensively studied and the issue of whether microvariants of EV can directly infect, replicate and persist in, and cause damage of β cells remains controversial (Flodstrom et al., 2002). More than three decade ago Yoon et al. showed that CVB4 is capable of replicating in cultured human islets (Yoon et al., 1978). Other works suggested that the CVB group has variants that can replicate in islet cells (Harting et al., 1983; Chatterjee et al.,1988), and by growing viruses on islets of Langerhans it is possible to isolate strains that can induce insulitis experimentally in animals and replicate in islet cells *in vivo*. Prototype CVB3, CVB4 and CVB5 as wells as CVA9 can replicate *in vitro* in purified insulin-producing β cells, and infection may result in functional impairment or cytolytic death of the β cells, but it may also have no apparent adverse effect (Roivainen et al., 2000; Roivainen et al., 2002). It appears that the consequences of the virus replication on β cell survival and function are not entirely dependent on the serotype but on a as-yet unidentified characteristics of the virus strain. For instance, between the diabetogenic strain E2 of CVB4 and the prototype CVB4, a 111 amino acid difference has been identified, and

amino acids or nucleotides potentially most critical for the pathogenesis of type 1 diabetes have to be identified amongst the microvariants of relevant virus strains.

Another work (Chehadeh et al., 2000) indicates that the CVB group is capable to replicate at a low level in human islet cells *in vitro,* persisting without cytolytic effect. This replication is associated with chronic synthesis of IFN-α by the islet cells. Neutralization of the IFN-α leads to a rise in viral replication and rapid islet destruction.

Type I interferons induce an anti-viral state in infected cells, providing an early defense against viral infections (Stark et al., 1998), and it appears that β cells may depend on interferons to lower their permissiveness to CVB4 infection, thus regulating the susceptibility to virus-induced diabetes (Flodstrom et a., 2002; Flodstrom et al., 2003). In this model, NOD mice that expressed the suppressor of cytokine signalling 1 (SOCS-1) in β cells developed diabetes, due to the replication of the virus in the β cells. A critical link between the target β cell antiviral responses and susceptibility to disease is thus established.

Furthermore, due to its immunoregulatory properties, IFN-α represents a link between the innate and the adaptive immunity: a pathological event may commence with activation of the innate immune system in order to avoid cytolytic destruction, followed by T cell activation and expansion, including autoreactive T cells. Viral expansion of non–specific T cell responses has been shown to be mimicked by injection of IFN-α (Tough & Sprent, 1996), or IFN-α expression by pancreatic β cells (Stewart et al., 1993; Chakrabarti et al., 1996). In humans, IFN-α has been detected in β cells (Foulis et al., 1987; Huang et al., 1995) and in blood of type 1 diabetic patients (Chehadeh et al., 2000).

A very recent report, indicates that rare genetic variations occurring in the gene IFIH1 and affecting the expression and structure of its protein product IFIH, lower the risk of developing type 1 diabetes (Nejentsev et al., 2009). IFIH1 triggers the secretion of interferons. Another study showed that IFIH1 expression in peripheral blood cells is associated with type 1 diabetes (Liu et al., 2009). These data allows to speculate that, on viral infection, interferon-response genes are activated in insulin-producing cells, leading to increased levels of interferons. Interferons inhibit viral replication, but also enhance the expression of surface MHC-I molecules. Cytotoxic CD8 T cells recognize infected β cells, through the MHC-I molecules, damaging and eventually killing them. Thus, viral infection can contribute to the development of type 1 diabetes.

As for i*n vivo* studies in humans, the isolation of an EV has been documented only few times. Historically, CVB4 was successfully cultured from the pancreas of a diabetic child at disease onset and it induced diabetes in susceptible animals, more than 30 years ago (Yoon et al., 1979). The diabetogenic E2 strain was likewise obtained by plaque purification of the human isolate Edwards of CVB4, that was isolated from a child with widespread CVB4 infection, presenting with acute myocarditis and pancreatitis (Chatterjee et al., 1988).

In contrast with the apparent success in the detection of EV mRNA from blood, no EV genome could be detected when pancreas from cases of type 1 diabetes were analysed post-mortem during the first year after diagnosis (Foulis et al., 1997). However, in the same study EV genome could be detected in the pancreas of children who died of acute myocarditis in which the heart was EV positive. These discrepancies may be explained with the argument that there is a critical window for virus detection in the pancreas, and this is only potentially achieved when there is an acute presentation of viral illness, as in the case of myocarditis. By *in situ* hybridisation studies on post-mortem pancreatic tissues of several type 1 diabetic patients, EV RNA positive cells were for the first time detected, and exclusively in islets (Ylipaasto et al., 2004).

In recent years, other studies have eventually indicated the presence of EV in pancreatic tissue in a sizable proportion of patients dying soon after diabetes onset (Tauriainen et al., 2009; Dotta et al., 2007; Richardson et al., 2009; Ylipaasto et al., 2004). A β cell infectious CVB4 was isolated in the pancreas of three patients at disease onset, by immunohistochemical, electron microscopy, genome nucleotide sequencing, cell culture and immunological studies (Dotta et al., 2007). Infection was specific to β cells, which showed islet inflammation mediated mainly by natural killer cells, reduced insulin secretion. The virus was also able to infect β cells from human islets of non diabetic donors.
A strain of echovirus 3 was isolated from an individual currently with appearance of islet cell and IA-2 autoantibodies (Williams at al., 2006). Richardson et al (Richardson et al., 2009) identified EV VP1 capsid protein in islets of 44 out of 72 recent-onset type 1 diabetic patients, and the staining was restricted to β cells. A recent report suggests that the virus is present in the intestinal mucosa of diabetic patients (Oikarinen et al., 2008).
These detection reports strengthen the case for a viral role in the pathogenesis of type 1 diabetes.

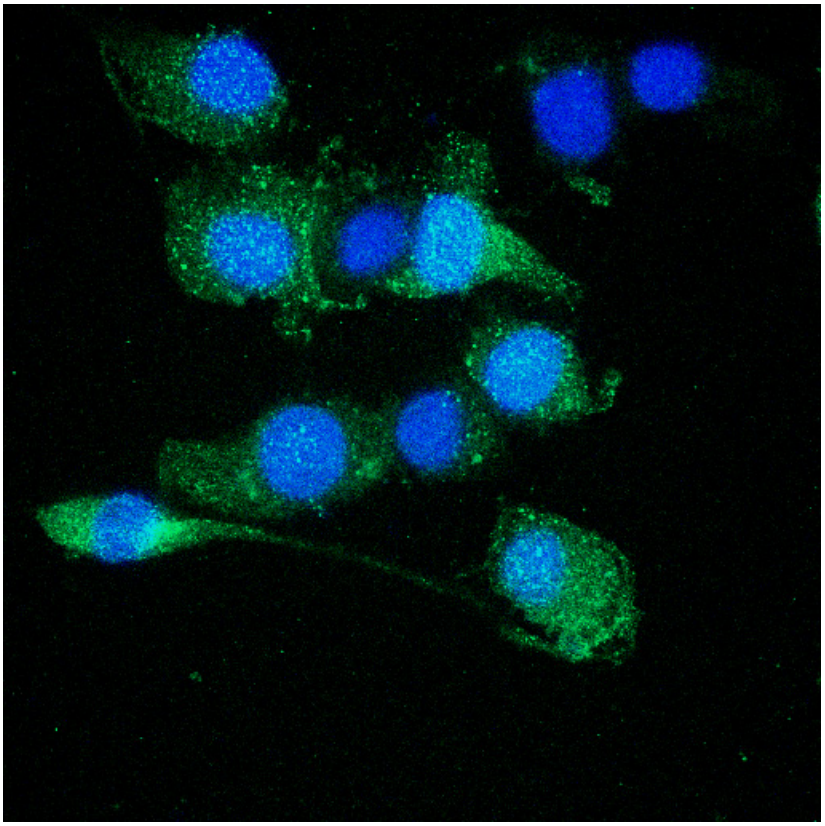

Fig. 3. Representative confocal immunofluorescence micrographs of islet MECs, stained with polyclonal anti-HCAR Ab, showing a diffuse expression in a fine punctate pattern in islet MECs (original magnification 630X, nuclei stained in blue with DAPI).

4.2 Coxsackievirus infection and endothelial cells

The host factors modulating viral infections include not only the host immune response, but also types and characteristics of cells that become infected in different tissues. Parenchymal cells of an organ are rarely in direct contact with the circulatory system, and viruses in the circulation must either circumvent or infect vascular endothelial cells to reach secondary organs. Vascular endothelial cells act in fact as important interface between the vascular space and the organ parenchyma, and, as previously stated, endothelial cells in different organs exhibit diverse structural and functional characteristics that can influence biological and pathological functions. Amongst these, vascular endothelial cells have an established role as mediators of tissue tropism and access for virus, influencing organ and tissue specific susceptibility to viral infection. Therefore, they can modulate the pathological expression of virus-induced diseases (Friedman et al., 1981; Huber et al., 1990; Conaldi et al., 1997; Zanone et al., 2003). For instance, in murine studies on CVB infectivity of different organs, CVB3 isolates from the heart showed greater infectivity and replication in heart endothelial cells than endothelial cells derived from liver or lung (Huber et al., 1990), thus confirming the essential role of host factors in developing specific diseases.

In line with this scenario, it is essential that, to gain access to secondary organs, viruses pass through the vascular endothelium by transcytosis or infection, or via infected circulating cells migrating into the target tissues. Endothelial cells derived from different organs show distinct susceptibility to CVB infections, and the behaviour against a viral challenge of endothelial cells in large vessels and microvessels may differ (Friedman et al., 1981; Huber et al., 1990; Conaldi et al., 1997; Zanone et al., 2003; Saijets et al., 2003). Endothelial cells expressing appropriate receptors would fail to act as effective barrier to infections, allowing viral particles to pass through, and replicate in, the vascular endothelium.

Human umbilical vein-derived endothelial cells have been shown to be persistently infected by different CVB strains (Flodstrom et al., 2000; Huber et al., 1990; Conaldi et al., 1997).

However, physiological and pathological events take place mainly at the level of the microvasculature. Using a dermal microvascular endothelial cell line, we have provided evidence that small vessel endothelial cells can harbour a persistent CVB viral infection (Zanone et al., 2003). All 3 CVB tested productively infected microvascular endothelial cells for up to 3 months without obvious cytolysis. A small proportion of the cells, approximately 10%, appeared to be involved in viral replication during chronic infection, suggesting that persistence is probably established through a mechanism of carrier-state culture, as proposed to explain CVB persistence in other cell types (Flodstrom et al., 2002; Greening et al., 2003). In addition, the infection increased production of proinflammatory cytokines IL-6 and IL-8, indicating endothelial cell activation by virus, and induced quantitative modification of adhesion molecule expression (ICAM-1, VCAM-1). These upregulation may influence the pattern of migration and extravasation of leucocytes in inflammation and immunity. These data add weight to the view that common CV infections are able to trigger complex pathophysiological processes, rather than simple cell lysis, as is becoming increasingly evident in clinical and experimental settings. These viruses can in fact persist for a considerable time in infected patients and cause chronic pathology or trigger immunopathological damage to infected and uninfected tissues (Muir et al., 1989; Stone, 1994). Furthermore, chronic infection of endothelial cells *in vivo* could provide better viral access to tissues underlying the endothelial layer and subsequent parenchymal cell infection.

The mechanisms of CVB persistence is not clear. It is possible that the infected cells undergo cytolysis and release virions to infect more cells, thus maintaining a chronic infection of the culture without massive cell destruction. Alternatively, it could be hypothesized that the cells can cure themselves of viruses, e.g. by limiting production of cell host products required for viral replication, or by production of anti-viral mediators. Stability of the cell membrane could also be another important factor in the ability of infected cells to survive infection, without lysis. In previous studies, the distinct susceptibility of different cell types to long-term infection has been related to the production of interferons (Conaldi et al., 1997**;** Heim et al., 1992).

It has also been suggested that the persistent infection of cultured HUVEC may be due to down-regulation of viral receptors in infected cells. However, a study indicates that the expression of the specific CVB receptor, CAR, in these cells was not quantitatively altered by infection with CVB but rather by culture confluence (Huber et al., 1990).

Human islet endothelial cells have been more recently shown to express the specific human Coxsackievirus and Adenovirus receptor (HCAR) (Figure 3) and CVB co-receptors, such as DAF, integrins and ICAM-1, that have differentiated functions on virus attachment and entry into target cells (Zanone et al., 2007). Islet endothelial cells can harbour a persistent, low level infection by CVB, assessed as detection of VP1 capsid protein and release of infectious particles. The infection has no obvious effects on cell morphology or viability and can provide better viral access to the underlying islet tissue. Under experimental conditions to avoid massive cytolysis and possibly to mimic silent *in vivo* infection, as EV infections can cause little or no clinical symptoms, only a proportion of cells appeared to be involved in viral replication, suggesting a mechanism of carrier-state culture (Conaldi et al., 1997; Zanone et al., 2003).

Notably, the infection of islet enodothelial cells upregulates the expression of DAF, HCAR and integrin $\alpha_v\beta_3$, in contrast to the behaviour of other macro- and microvascular endothelial cells lines, i.e. HUVEC, HMEC-1 and human aortic ECs. In fact, it has been shown that CVB infection downregulates DAF on HUVEC and HMEC-1 (Zanone et al., 2003), leaves HCAR expression unchanged on HUVEC (Carson et al., 1999), and downregulates HCAR expression and upregulates DAF expression on human aortic endothelial cells (Zanone et al., 2007).

This differential behaviour underlines the widely accepted heterogeneity of phenotype and function amongst endothelial cells derived from different vascular beds (Swerlick et al., 1992; Lidington et al., 1999), and may be relevant for the pathological sequelae of the infection. Despite detailed knowledge of the molecular structure and virus interaction of HCAR, its biological and possible pathogenic relevance are uncertain. HCAR belongs to the immunoglobulin superfamily and appears to have signalling functions (Bergelson et al., 1997; Noutsias et al., 2001; Fechner et al., 2003). Remarkably, CAR has been shown to be upregulated on affected cardiomyocytes in a rat model of experimental autoimmune myocarditis (Ito et al., 2000) and in human idiopatic dilated cardiomyopathy (Noutsias et al., 2001), for which EVs are the most frequently implicated pathogens (Feldman, 2000). CAR expression could therefore represent a key determinant of cardiac susceptibility to viral infections and have a pathogenic relevance in chronic cardiomyopathies. It has also been suggested that cell-to-cell contact modulates CAR-to-CAR interaction-based signals (Carson et al., 1999**;** Fechner et al., 2003).

The CVB infection upregulates in islet endothelial cells the expression of adhesion molecules and increases the production of proinflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8 as well as IFN-α, once again pointing to endothelial cell activation (Zanone et al., 2007), in line with studies that suggest that a low-grade inflammation may cause profound impairments of endothelial function (Hingonari et al., 2000; Charakida et al., 2005).
In time course analyses, infected cells transiently upregulated expression of two major adhesion molecules, which may have *in vivo* functional consequences, enhancing cellular recruitment and leading to persistent tissue inflammation.

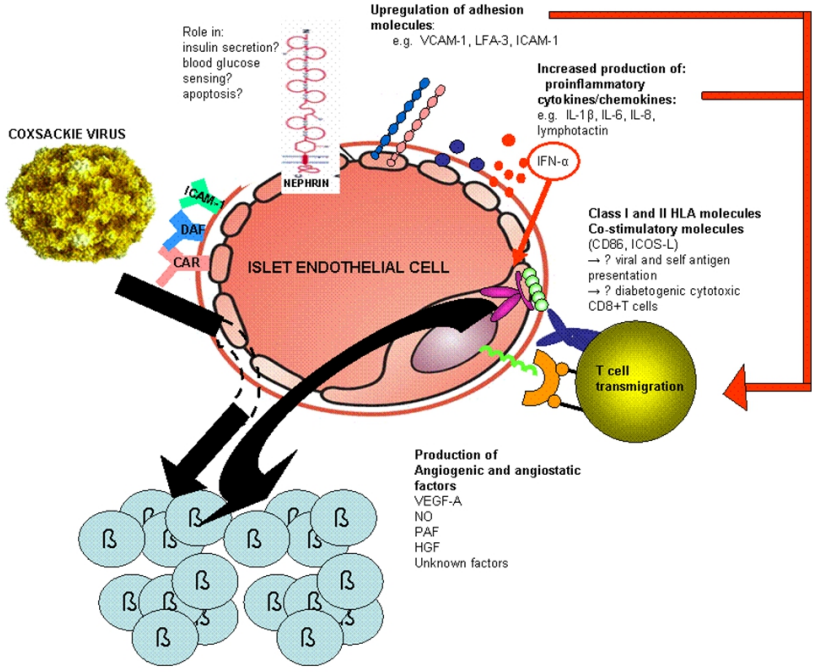

Fig. 4. Schematic representation of the relationship between Coxsackievirus infection, islet endothelial cells and β cells.
The highly fenestrated endothelial cells exhibit expression of classical endothelial markers, adhesion and co-stimulatory molecules, with the potential of being involved in autoreactive T cell adhesion, activation and transmigration in type 1 diabetes. They express specific markers, such as nephrin, whose functions at this site remain to be unravelled. Human islet endothelial cells express receptors and coreceptors for Coxsackievirus (such as HCAR, DAF, integrin and ICAM-1). These cells are potential target of an acute or persistent CVB infection, that activates the endothelium, upregulates expression of adhesion molecules, increases the production of proinflammatory cytokines and chemokines, and provides better viral access to tissues underlying the endothelial layer. Moreover, the increased production of IFN-α may enhance the expression of surface class I and II HLA molecules involved in viral and self antigen presentation, with selective recruitment and expansion of cytotoxic CD8+ T cells, which recognize infected endothelial and β cells, eventually damaging and killing them.

Infection also increased the production of pro-inflammatory cytokines, IL-1β, IL-6 and IL-8, further contributing to viral pathogenetic sequelae and to an indirect amplification of virus specific and non-specific responses. In this scenario, an exacerbated local inflammatory response secondary to viral infection represents an attempt to restrict virus replication, but it could promote chemoattraction and homing of circulating viral or, in susceptible individuals, islet antigen-specific T cells, in a bystander activation model (Horwitz et al., 1998). Cytokines may also be directly toxic to β cells, leading to release of sequestered antigens, presentation by professional dendritic cells, and activation of autoantigen-specific T cells. Endothelial cells themselves may serve as antigen-presenting cells (Greening et al., 2003; Savinov et al., 2003).

The infection was also accompanied by increased production of IFN-α, that has a role in initiation and maintenance of chronic CVB infection, as shown for other infected cell lines including islet β cells, and in line with the extensive studies documenting abnormal localization of IFN-α in the pancreas of type 1 diabetic patients (Chehadeh et al., 2000, Huber et al., 1990, Conaldi et al., 1997; Heim et al., 1992). As stated above, IFN-α may be responsible for a viral expansion of non–specific T cell responses, including autoreactive T cells.

Again, in dilated cardiomyopathy inflammatory endothelial activation is present, and endothelial CAM expression correlates with the intramyocardial counterreceptor-bearing lymphocyte infiltrates (Noutsias et al., 1999; Seko et al., 1993). In this model, it is likely that endothelial cells are infected before cardiotropic viruses invade the myocardium (Klingel et al., 1992).

An increased production of lymphotactin RNA by the infected cells is also reported. Lymphotactin is a chemokine with the ability to chemoattract highly specifically $CD4^+$ and $CD8^+$ T cells and NK cells (Kennedy et al., 1995; Hedrick et al., 1997), with possible anti-viral and anti-tumor effects. An inappropriate T cell infiltration, drawn by lymphotactin, is present in other inflammatory conditions (Middel et al., 2001; Blaschke et al., 2003), and lymphotactin exposed on infected islet endothelial cells could, therefore, play a role in islet infiltration by T cells.

The endothelium infection may thus contribute to selective recruitment and expansion of subsets of leukocytes during inflammatory immune responses in type 1 diabetes. These findings add to a body of work that highlights the possible role of human EVs as environmental triggers that are capable of influencing the incidence of type 1 diabetes, the susceptibility of which to environmental influences is well established.

5. Conclusion

There is a body of work that highlights the possible role of human EVs as environmental triggers that are capable of influencing the incidence of type 1 diabetes, the susceptibility of which to environmental influences is well established (Hyöty & Taylor, 2002; Varela-Calvino & Peakman, 2003). Vascular endothelial cells have a major role in viral tropism and disease pathogenesis. Islet endothelium appears to be endowed of distinctive structural and functional features, and is acquiring a role in type 1 and type 2 diabetes (Figure 4). An interaction between islet endothelium and an EV, CVB in particular, infection might trigger a series of pro-inflammatory events that could be important in islet inflammation and possibly influence the development of autoimmune diabetes, through the initiation or acceleration of islet autoimmunity in susceptible individuals.

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4

Type 1 Diabetes Mellitus and Co-Morbidities

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1. Introduction

Co-morbid conditions are relatively frequent in Type 1 Diabetes Mellitus (T1DM). They can severely affect clinical management of the disease, especially in pediatric age.
Furthermore, these conditions could present very interesting ethiopatogenetic mechanisms.

2. Associated autoimmune conditions

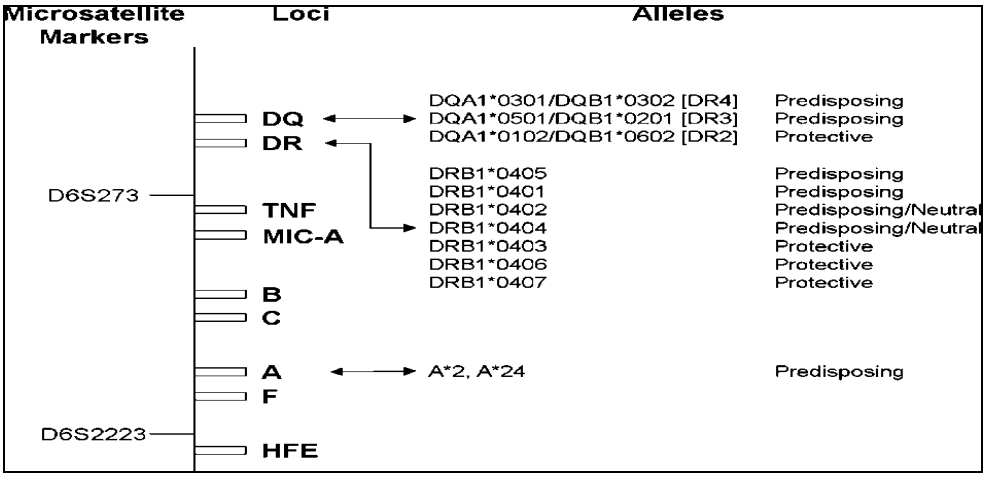
2.1 Genetic associations

Patients with type 1 diabetes (T1D) have an increased risk of other autoimmune conditions, such as autoimmune thyroid disease (AIT), celiac disease (CD), Addison's disease (AD) and vitiligo. These diseases are associated with organ-specific autoantibodies: AIT with thyroid peroxidase (TPO) and thyroglobulin autoantibodies (TG), CD with endomysial (EMA) and transglutaminase (TTG) autoantibodies, and AD with adrenal autoantibodies. Using these autoantibodies, organ-specific autoimmunity may be often detected before the development of clinical disease, in order to prevent significant morbidity related to unrecognized disease (Barker, 2006). The probable mechanism of these associations involves a shared genetic background (Myśliwiec et al., 2008; Smyth et al., 2008).
The majority of autoimmune endocrinopathies, including T1D, are inherited as complex genetic traits. Multiple genetic and environmental factors interact with each other to confer susceptibility to these disorders. Genetic risk factors associated with T1D, ATD, CD and AD include HLA genes and non-HLA genes.

2.1.1 HLA genes

The major histocompatibility complex (MHC) has been extensively studied in these diseases. HLA molecules are highly polymorphic and multiple different peptides can be presented to T cells by these molecules. In general it appears that the alleles associated with autoimmunity are not abnormal, but functional variants, that aid in determining specific targets of autoimmunity. The leading hypothesis is that these molecules contribute to determine risk through the peptides they bind and present to T-lymphocytes, either by influencing thymic selection, or peripheral antigen presentation. (Ide & Eisenbarth, 2003).
HLA DR4 and DR3 are strongly associated with T1D and approximately 30-50% of patients are DR3/DR4 heterozygotes. The DR3/DR4 genotype confers the highest diabetes risk with a synergistic mode of action, followed by DR4 and DR3 homozygosity, respectively. The

HLA-DQ (particularly DQ 2 and DQ8) locus has been found to be the most important determinant of diabetes susceptibility. Approximately 90% of individuals with T1D have either DQ2 or DQ8, compared to 40% of the general population (Ide & Eisenbarth, 2003). So, the highest-risk human leukocyte antigen (HLA) genotype for T1D is DR3-DQ2, DR4-DQ8.
DR3-DQ2 shows a strong association with CD; homozygosity for DR3-DQ2 in a population with T1D carries a 33% risk for the presence of TTG autoantibodies (Bao et al., 1999). Moreover, in families with multiple members affected with T1D and AIT, DR3-DQ2 has been linked with AIT and T1D (Levin et al, 2004). AD has been associated with the presence of a rare subtype of DR3-DQ2, DR4-DQ8 in which the DR4 subtype is DRB1*0404. This subtype is found in less than 1% of the general population compared with 30% of the population with AD (Barker et al., 2005; Myhre et al., 2002; Yu et al., 1999). A schematic representation of the HLA region and its association with T1D is shown in the Figure 1.



(from Pugliese A. and Eisenbarth G.S., Chapter 7, Type 1 Diabetes: Molecular, Cellular, and Clinical Immunology, www.barbaradaviscenter.org)

Fig. 1. The HLA Region and T1D susceptibility. Schematic representation of the HLA region showing microsatellite markers, loci, and alleles associated with T1D susceptibility. Distances between loci are grossly approximated.

2.1.2 Non-HLA genes

Non-HLA genes are also involved in the predisposition to T1D and other autoimmune diseases, such as MIC-A, PTPN22, CTLA-4 (Barker, 2006).
Polymorphisms of MIC-A (MHC I-related gene A) have been associated with T1D, CD and AD. This gene encodes for a protein that is expressed in the thymus and interacts with the receptor NKG2D, which is important for thymic maturation of T cells (Hue et al., 2003). It is hypothesized that the loss of this interaction is a way in which immunological tolerance may be lost. NKG2D also regulates the priming of human naïve CD8+ T cells, providing an alternative explanation for associations with autoimmune diseases (Maasho et al., 2005).
The PTPN22 gene is expressed in T cells and encodes lymphoid tyrosine phosphatase (LYP). LYP appears to be important in the signal cascade downstream from the T-cell receptor. A

specific polymorphism, changing an arginine to tryptophan at position 620, has been associated with T1D (Bottini et al., 2004; Smyth et al., 2004) and also other autoimmune disorders, such as rheumatoid arthritis, systemic lupus erythematosus, Graves' disease and weakly with AD. The association with many autoimmune diseases suggests that this gene may be playing a role in susceptibility to autoimmunity in general.
Another non-HLA gene associated with T1D which has a generic role in susceptibility to autoimmunity is CTLA-4 (Cytotoxic T lymphocyte-associated antigen-4) (Vaidya & Pearce, 2004). CTLA-4 gene is an important susceptibility locus for autoimmune endocrinopathies and other autoimmune disorders, including T1D (Ueda et al., 2003). The CTLA-4 gene, which is located on chromosome 2, encodes a costimulatory molecule that is expressed on the surface of activated T cells. It plays a critical role in the T-cell response to antigen presentation, binding costimulatory molecules and inhibiting T-cell activation. (Vaidya & Pearce, 2004). The inhibitory effect of CTLA-4 on T-cell activation has led the investigations into its role in different human autoimmune disorders. Polymorphisms within the CTLA-4 gene have been linked to AIT (Vaidya et al., 1999). CTLA-4 has also been linked to AD and more strongly to subjects affected by AD in association with T1D and AIT compared with AD alone (Vaidya et al., 2000). CTLA-4 has been associated with a wide range of other autoimmune disorders, including primary biliary cirrhosis, multiple sclerosis, CD and rheumatoid arthritis. These observations have suggested that CTLA-4 is a general autoimmune locus, and that the susceptibility polymorphisms within the gene may lead to general defects in the immune regulation, while other tissue-specific (e.g. insulin gene polymorphisms) or antigen-specific (e.g. MHC) genetic factors and environmental factors determine the involvement of particular target organs (Vaidya & Pearce, 2004).

Gene	Associated diseases
MIC-A	T1D, CD, AD
PTPN22	AIT, AD
CTLA-4	T1D, AIT

Table 1. Non-HLA genes associated with T1D and other autoimmune diseases

2.2 Type 1 diabetes and celiac disease

2.2.1 Prevalence and age at starting

Traditional studies, both in children and adults, have shown that CD occurs in patients with T1D with a prevalence that varies from 1,5 to 10 % compared with 0.5 % of the general population (Cronin & Shanahan, 2007; Vaarala, 2000). The mean age at diagnosis of classical CD is commonly around 2-3 years, while the mean age at diagnosis of DM1 is 7-8 years. The age at onset of T1D is younger in patients with the double disease than in those with only T1D (Kaspers et al., 2004). The risk of CD is negatively and independently associated with age at onset of diabetes, with an higher risk being seen in children age < 4 years than in those age > 9 years (Cerutti et al., 2004). In patients with T1D, diabetes is usually diagnosed first, CD precedes diabetes onset only in 10-25% (Cerutti et al., 2004; Valerio et al., 2002), while generally CD diagnosis in T1D patients occurs, trough the screening performed at diabetes onset, in 70-80% of patients with a median age >8 years. Some authors hypothized that in genetically susceptible patients one disease could predispose to another. Particularly, it has been suggested that untreated (latent or silent) CD could be an immunological trigger and induce diabetes and/or thyroid disorders due to gluten as a driving antigen (Pococco &

Ventura, 1995). In accordance with this, the prevalence of autoimmune disorders in CD is closely related to age at diagnosis or, in other words, to the duration of exposure to gluten (Ventura et al., 1999) and thyroid-related antibodies tend to disappear during twelve months of gluten-free diet, like CD-related antibodies (Ventura et al., 2000). However, at present, it is unknown whether treatment of CD reduces the likelihood of developing autoimmune disorders, or changes their natural history and actually others found no correlation between duration of gluten exposure in adult CD and risk of autoimmune disorders (Viljamaa et al., 2005).

2.2.2 Clinical features and follow up

The classic presentation of CD describes symptoms related to gastrointestinal malabsorption and includes malnutrition, failure to thrive, diarrhea, anorexia, constipation, vomiting, abdominal distension, and pain. This predominance of gastrointestinal symptoms is more common in children younger than three years of age. Non-gastrointestinal or atypical symptoms of CD include short stature, pubertal delay, fatigue, vitamin deficiencies, and iron deficiency anemia and are more commonly observed in older children. The classical presentation of CD can occur in T1D patients, but many patients with CD and T1D are either asymptomatic (silent CD) or present with only mild symptoms (Holmes, 2001a; Ventura et al., 2000). Diagnosis of CD is regularly performed because screening protocols are universally recommended and performed. In patients with overt CD, identifying and treating CD with gluten free diet (GFD) surely confer benefit in reducing complications such as malabsorption, infertility, osteoporosis, poor nutrition, impaired growth and reducing long-term malignancy risks and mortality rates (Collin et al., 2002; Freemark & Levitsky, 2003; Rubio-Tapia et al., 2009), while no evidence exists on long-term morbidity in silent CD. Similarly, children with T1D with evidence of symptomatic CD benefit from GFD (Hansen et al., 2006; Saadah et al., 2004); in symptom-free cases the demonstrated benefit is limited to weight gain and bone mineral density (BMD) changes.(Artz et al., 2008; Rami et al., 2005; Simmons et al., 2007). Recently a 2-year prospective follow up study has provided additional evidence that only in some of the children with T1D and few classical symptoms of CD, identified by screening as being TG+ present, the demonstrated benefit of GFD is limited to weight gain and BMD changes (Simmons et al., 2011); moreover, other authors have reported an improved glycemic control in GFD-compliant celiac patients (Sanchez-Albisua et al., 2005). On the contrary, silent untreated CD has no obvious effect on metabolic control in T1D patients, but could negatively influence weight gain (Rami et al., 2005). In any case, the adherence to GFD by children with T1D has been reported generally below 50% (Acerini et al., 1998; Crone et al., 2003; Hansen et al., 2006; Saadah et al., 2004, Westman et al., 1999). The different viewpoints highlight the need of a long follow up of patients affected by T1D and asymptomatic CD to clarify the role of a GFD. Actually some authors argument against the need to stress GFD in nonsymptomatic T1D patients (Franzese et al., 2007; Van Koppen et al., 2009). However, the wide spectrum of CD include also subjects with positive celiac-related antibodies without diagnostic small-bowel mucosal villous atrophy. This condition is defined as potential celiac disease (pot-CD) (Holmes, 2001b; Paparo et al., 2005; Troncone et al., 1996). Some authors described that the prevalence of pot-CD among patients with T1D recruited from the majority of childhood diabetes care centers in Italy is 12.2 %, with an higher prevalence of females. The prevalence of pot-CD in the CD control population is 8.4 % (Franzese et al., 2011). Case reports and small follow-up studies indicated that only few pot-CD patients may suffer from CD-related symptoms

before the development of villous atrophy (Troncone et al., 1996). No definite consensus exists among experts about to treat pot-CD patients with GFD. No data are available on the natural history of these patients in the long term, nor on the risks they are exposed if left on normal gluten-containing diet, while a recent paper provided evidence that pot-CD children may benefit from GFD treatment (Kurppa et al., 2010).
Other studies have shown intestinal inflammation also in T1D patients without CD-related antibodies and structurally normal intestinal mucosa (Westerholm-Ormio et al., 2003). According to this, our group has observed a gluten-related inflammation either in rectal either in small bowel mucosa of children with T1D (Maglio et al,. 2009; Troncone et al., 2003). It can be speculated that gluten could be an optimal candidate to stimulate an abnormal innate immune reaction in intestinal mucosa due to its pro-inflammatory characteristics. It remains a crucial issue to estabilish to what the extented intestinal inflammation in T1D is gluten-dependent and whether it precedes the occurrence of the disease.

2.3 Type 1 diabetes and autoimmune thyroid disease

2.3.1 Prevalence and age at starting

Antithyroid antibodies have been shown to occur during the first years of diabetes in 11-16.9% of individuals with T1D (Kordonouri et al., 2002). Long-term follow up suggests that as much as 30 % of patients with T1D develop AIT (Umpierrez et al., 2003). The range of prevalence of AIT in patients with T1D is unusually wide (3.4-50%) (Burek et al., 1990; Radetti et al., 1995). Thyroid antibodies are observed more frequently in girls than in boys, often emerging along during pubertal maturation (Kordonouri et al., 2005).

2.3.2 Clinical features and follow-up

Hyperthyroidism is less common than hypothyroidism in association with T1D (Umpierrez et al., 2003), but still more common than in the general population. It may be due to Grave's disease or the hyperthyroid phase of Hashimoto's thyroiditis. The presence of abnormal thyroid function related to AIT in the population with T1D has the potential to affect growth, weight gain, diabetes control, menstrual regularity, and overall well-being. In particular clinical features of hypothyroidism may include the presence of a painless goitre, increased weight gain, retarded growth, tiredness, lethargy, cold intolerance and bradycardia while diabetic control may not be significantly affected. Clinical features of hyperthyroidism may include unexplained difficulty in maintaining glycaemic control, weight loss without loss of appetite, agitation, tachycardia, tremor, heat intolerance, thyroid enlargement or characteristic eye signs. The treatment of hypothyroidism is based on replacement with oral L-thyroxine (T4) sufficient to normalise TSH levels and usually this allows regression of the goitre if present. The treatment of hyperthyroidism is based on the use of carbimazole and beta-adrenergic blocking drugs, if necessary.
There are studies showing worse diabetes control in patients with a second autoimmunity, including AIT and CD (Franzese et al., 2000; Iafusco et al., 1998). The factors responsible for the worsened control have not been completely elucidated. Thyroid dysfunction could be responsible of variations in absorption of carbohydrates and increased insulin resistance. There are studies showing similar diabetes control in patients with and without a second autoimmunity, in these studies thyroid autoimmunity does not lead to worsening of diabetic metabolic control in children with T1D (Kordonouri et al., 2002; Rami et al., 2005; Sumnik et al., 2006). The thyroid status is not different between diabetic patients with and

without CD: children with both T1D and CD do not have an increased risk of AIT development compared to diabetic patients without CD (Sumnik et al., 2006).

2.4 Type 1 diabetes, Addison disease and polyglandular syndromes

2.4.1 Prevalence and age at starting

Addison's disease (AD) affects approximately 1 in 10,000 of the general population. The autoimmune process resulting in AD can be identified by the detection of autoantibodies against the adrenal cortex (Anderson et al., 1957; Lovas & Husebye, 2002). Up 2 % of patients with T1D have antiadrenal autoantibodies (De Block et al.; 2001, Falorni et al., 1997; Peterson et al., 1997).

AD is occasionally associated with T1D in the Autoimmune Polyglandular Syndromes (APS I and II). APS I, also known as autoimmune polyendocrinopathy candidiasis ectodermal dysplasia (APECED), is a rare polyendocrine autoimmune disease caused by mutations of the autoimmune regulator gene (AIRE) on chromosome 21q22.3 (Aaltonen et al., 1994; Ahonen et al., 1990), which is characterized by the association of mucocutaneous candidiasis, adrenal insufficiency, and/or hypoparathyroidism. Follow-up of subjects with this disorder has revealed that many organ systems may be involved in the autoimmune process including the pancreatic β cell. Approximately 20% of subjects with APS-I develop T1D (Barker, 2006). APS II is more common in adults, but is also observed in children in association with autoimmune thyroiditis (Dittmar & Kahaly, 2003). Other less common disorders observed in APSII include Addison's disease, hypogonadism, vitiligo, alopecia, pernicious anemia and myasthenia gravis. Another rare disorder associated with T1D in early childhood is the Immunodysregulation Polyendocrinopathy X-linked Syndrome (IPEX), which is characterized also by severe enteropathy and autoimmune symptoms due to a clear genetic defect (FOX-P3) (Chatila et al., 2000). FOX-P3 is expressed in CD4+CD25+ regulatory T cells; mutations result in the inability to generate these regulatory T cells resulting in multiorgan autoimmunity (Barker, 2006).

2.4.2 Clinical features and follow-up

The condition of AD is suspected by the clinical picture of frequent hypoglycaemia, unexplained decrease in insulin requirements, increased skin pigmentation, lassitude, weight loss, hyponatraemia and hyperkalaemia. The diagnosis is based on the demonstration of a low cortisol, especially in response to ACTH test. Treatment with a glucocorticoid is urgent and life-threatening. In some cases the therapy has to be supplemented with a mineralocorticoid. In asymptomatic children with positive adrenal antibodies, detected on routine screening, a rising ACTH level suggests a failing adrenal cortex and the development of primary adrenal insufficiency (Kordonouri et al., 2009). There are no current recommendations for screening of adrenal autoimmunity.

2.5 Type 1 diabetes and vitiligo

Vitiligo is an acquired pigmentary disorder characterized by a loss of melanocytes resulting in white spots or leukoderma. The association of vitiligo with other autoimmune disorders, including thyroid disease, adrenal insufficiency, gonadal dysfunction, polyendocrine failure, diabetes mellitus, pernicious anemia, myasthenia gravis and alopecia areata, has been well documented (Bystryn, 1997; Handa & Dogra, 2003). This condition is present in about 6% of diabetic children (Hanas et al., 2009). Spontaneous re-pigmentation is rare and

not usually cosmetically acceptable. Treatment is difficult and multiple therapies have been tried with little success . (Ho et al., 2011)

2.6 Type 1 diabetes and collagenopathies

2.6.1 Rheumatoid arthritis

The tendency of autoimmune diseases to aggregate is well known as clusters of autoimmune diseases within families and individuals. Analysis of susceptible genetic loci for the distinct autoimmune disease shows considerable overlap that suggests the possibility of shared pathways in their pathogenesis. Reports on the clustering of T1D, AIT, CD and rheumatoid arthritis (RA) in the same patient are very scarce. The major genetic predisposition to RA is contributed by variants of the class II HLA gene, HLA DRB1. In exploring the overlap between T1D, CD and RA, there is strong evidence that variation within the TAGAP gene is associated with all three autoimmune diseases. Relatively little is known about the TAGAP gene, which encodes a protein transiently expressed in activated T cells, suggesting that it may have a role in immune regulation. So the TAGAP gene, previously associated with both T1D and CD, is also associated with RA susceptibility. Interestingly a number of loci appear to be specific to one of the three diseases currently studied suggesting that they may play a role in determining the particular autoimmune phenotype at presentation (Eyre et al., 2010). The majority of the published case reports are girls. The predominance of females among the affected individuals may reflect that certain genes play role in the pathogenesis as gender-specific factors or the penetrance of multiple risk genes are enhanced in females. In most reported patients, diabetes is diagnosed first, thyroid autoimmunity and juvenile rheumatoid arthritis develop after a period of several months to years. (Nagy et al., 2010; Pignata et al., 2000; Valerio et al., 2000).

2.6.2 Sclerodermia, systemic lupus erythematosus

The association of T1D with Systemic Lupus Erythematosus (SLE) and Sclerodermia is rare but reported in literature (Inuo et al., 2009, Zeglaoui et al., 2010). Some authors found a significant association between DQ2 allele and the presence of anti-SSA antibodies, while others described an association between CD and the presence of A1B8DR3 haplotype, which seems to be frequent in SLE and in Sclerodermia (Black et al., 1983; Mark, 2000; Sollid & Thorsby, 1993). In human, the CTLA-4 and PD-1 genes significantly contributed to the development of various autoimmune diseases in different genetic backgrounds (Inuo et al., 2009).). It has been suggest the involvement of CTLA-4 and PD-1 (inhibitor receptors of CD28) to the development of T1D, SLE or other autoimmune diseases.

Juvenile sclerodermia is present in 3% of sclerodermia cases, SLE in children is present in 9% of cases of SLE; one case of a 15 years girl with CD and SLE and Sclerodermia has been reported (Zeglaoui et al., 2010).

2.7 Screening for associated autoimmune disorders

Since Type 1 Diabetes is associated with the presence of additional autoimmune disease, such as AIT, CD and AD, which are associated with the production of organ-specific antibodies, it is possible to screen patients with T1D by means of these ones. However, only a subset of the subjects with organ-specific antibodies develops clinical disease. The frequency of screening and follow up of patients with positive antibodies remain controversial. The current American Diabetes Association (ADA) recommendations are to

screen for CD-associated antibodies at diagnosis of T1D and in presence of symptoms. The International Society of Pediatric Adolescent Diabetes (ISPAD) recommends to screen for CD at the time of diagnosis, annually for the first five years and every second year thereafter. More frequent assessment is indicated if the clinical situation suggests the possibility of CD or the child has a first-degree relative with CD. Respect to the screening for thyroid disease, current recommendations from the ADA are for screening TSH after stabilization at onset of diabetes, with symptoms of hypo- or hyperthyroidism, and every 1–2 yr thereafter. ISPAD recommends to screen by circulating TSH and antibodies at the diagnosis of T1D and, thereafter, every second year in asymptomatic individuals without goitre or in the absence of thyroid autoantibodies. More frequent assessment is indicated otherwise, subjects with positive TPO autoantibodies and normal thyroid function are screened on a more frequent basis (every 6 months to 1 yr). There are no current recommendations for screening of adrenal autoimmunity (Barker, 2006). Authors observed that the prevalence of adrenal antibodies in diabetic patients with thyroid antibodies compared with those without thyroid antibodies is increased (5,1 vs 0,6%) (Riley et al., 1981). It is possible conclude that routine screening for AD in children with T1D is not warranted unless there is a strong clinical suspicion or family history of AD (Marks et al., 2003)

Celiac disease	Transglutaminase antibodies	Yearly
Thyroiditis	TSH, FT4, thyroid antibodies	Yearly
Addison disease	Cortisolemia, adrenal antibodies	Screening if AD in family
Collagenopathies	Specific auto-antibodies	No screening

Table 2. Autoimmune diseases associated with T1D, recommended systems and frequency of the screening

3. Associated non-autoimmune conditions

3.1 Type 1 diabetes and growth

Type 1 diabetes and other chronic diseases are well known to adversely affect linear growth and pubertal development, this can include a wide spectrum of different conditions, from poor gain of weight to Mauriac Syndrome (MS); MS classically involves hepatomegaly, growth impairment, and Cushingoid features in poorly controlled diabetic patients. Although MS, the most important expression of growth alteration due to severe insulin deficiency in diabetic patients, is now rare, impaired growth in children with T1D is still reported. This is particularly true in patients with poor metabolic control (Chiarelli et al., 2004; Franzese et al., 2001). Some studies report that poorly controlled patients show a decrease in height standard deviation score over the next few years, while better controlled patients maintain their height advantage (Gunczler & Lanes, 1999; Holl et al., 1998).

Longitudinal bone growth is a complex phenomenon involving a multitude of regulatory mechanisms strongly influenced by growth hormone (GH) (Chiarelli et al., 2004) and by the interaction between insulin-like growth factors (IGF-I and IGF-II), that circulate bounded to specific insulin-like growth factor binding proteins (IGFBPs). IGFBP-3, the major circulating binding protein during post-natal life, is GH-dependent. Insulin is an important regulator of this complex. In fact, adequate insulin secretion and normal portal insulin concentrations are

needed to support normal serum concentrations of IGFs and IGFBPs and indirectly to promote growth. Poor gain of height and weight, hepatomegaly, non alcoholic steatosis hepatis (NASH) and late pubertal development might be seen in children with persistently poorly controlled diabetes. Similar to healthy adolescents, the pubertal growth spurt represents the most critical phase for linear growth and final height in children with T1D. The pubertal phase is characteristically associated with reduction in insulin sensitivity, which is known to be more severe in patients with T1D, and might negatively influence growth and height gain (Chiarelli et al., 2004). Although the chronological age at onset of puberty and the duration of the pubertal growth spurt is not significantly different between subjects with T1D and healthy adolescents, several studies have shown a blunted pubertal growth spurt which seems to be associated with a reduced peak of height velocity SDS (Vanelli et al., 1992). Although loss of height from the onset of diabetes has been widely reported, an impaired final height has not been reported in children with T1D. In fact, while some studies, especially those performed in the pre-intensive insulin therapy era, showed an impaired final height in children with diabetes (Penfold et al., 1995), more recent studies show a normal or only slightly reduced final height (Salerno et al., 1997).
The Diabetes Control and Complications Trial (DCCT) and other studies have reported increased weight gain as a side effect of intensive insulin therapy with improved metabolic control (DCCT Research Group, 1993). As obesity is a modifiable cardiovascular risk factor, careful monitoring and management of weight gain should be emphasised in diabetes care. Girls seem to be more at risk of overweight and as well of eating disorders.
Monitoring of growth and development and the use of percentile charts is a crucial element in the care of children and adolescents with diabetes. Improvements in diabetes care and management and especially newer insulin schedules based on multiple daily injections or insulin pumps have led to a reduction in diabetic complications and seem to ameliorate growth in children with T1D. Start an intensive insulin regimen since the onset of diabetes might prevent the induction of abnormalities of the GH–IGF-I–IGFBP-3 axis potentially achieving near-normal portal insulin concentrations and thereby leading to normal IGF-I and IGFBP-3 levels and physiological growth in children and adolescents with T1D.

3.2 Type 1 diabetes and eating disorders

Eating disorders (EDs) are a significant health problem for many children and adolescents with T1D similar to that observed in other high risk groups, such as competitive athletes, models and ballet dancers. EDs and subclinical disordered eating behaviors (DEBs) have been described in adolescents with T1D with a higher prevalence than in a non-diabetic population. The start of insulin treatment and the need to comply with dietary recommendations both lead to weight gain, which in turn leads to body dissatisfaction and a drive for thinness. Since the dietary restraint usually requires ignoring internal cues of hunger and satiety, it has been suggested that it may be a triggering factor in the development of cycles of binge eating and purging. The concurrence of T1D and EDs can greatly increase morbidity and mortality. In diabetic subjects, EDs are associated with insulin omission for weight loss and impaired metabolic control. On the contrary, in a five year longitudinal study, the expected relationship between ED and poor metabolic control was not evident, although there was a trend for higher haemoglobin A1c in individuals with an EDs (Colton et al., 2007). This offers hope that early interventions might prevent the worsening metabolic control that is often associated with EDs. In addition subclinical DEBs

among youth with T1D have been associated with increased risk of poor metabolic control and increased prevalence of microvascular complications such as retinopathy and nephropathy (Rydall et al., 1997). Some studies have examined the prevalence of EDs and DEBs in youth with T1D. Prevalence rates vary considerably from study to study possibly due to differences in sample, screening tools, and data collection methods. In a multi-site, cross sectional case-control study, the prevalence of ED meeting DSM-IV diagnostic criteria was about 10% and that of their sub-threshold variants about 14%: both were about twice as common in adolescent females with T1D than in their non-diabetic peers. (Jones et al., 2000). However there are also rare cases in childhood (Franzese et al., 2002a).

3.2.1 Management

Nutritional treatment is one of the main difficulties in managing diabetes in the young. Diabetes clinicians should be aware of the potential warning signs in an adolescent with diabetes as well as assessment and treatment options for eating disorders with concomitant T1D. Clinical approaches should focuse on normalizing eating behaviour and enhancing self-esteem based on personal attributes unrelated to weight and eating, with a low threshold for referral for specialized EDs services (Colton et al., 2007). A multidisciplinary team, composed by clinicians, psychologist/psychiatric, dietitian/nutrition therapist, especially one with a background in EDs, is opportune to identify and treat unhealthy EDs and DEBs in T1D. Treatment for adolescents with T1D should include both diabetes management treatment and mental health treatment. The diabetes team and the mental health team have separate responsibilities but work collaboratively to address disordered eating in patients with T1D. Treatment begins with emphasis on nutritional rehabilitation, weight restoration, and adequate diabetes control. Psychotherapy should begin immediately for the patient and family (S.D. Kelly et al., 2005).

3.3 Necrobiosis lipoidica diabeticorum

Necrobiosis lipoidica diabeticorum (NBL) is an infrequent skin affection in pediatric age. The etiology is not clearly understood. The reported prevalence in children varies from 0.06% to 10% (De Silva et al., 1999). The female/male ratio is 3:1(Hammami et al., 2008). The average age of onset is 30–40 years. In the past, it has been described as a complication of diabetes and associated with microvascular complications (W.F. Kelly et al., 1993), but NBL has been observed also at the beginning of diabetes. NBL typically appears on the anterior lower legs. The lesions are usually bilateral and are characterized by well circumscribed yellow brown inflammatory plaques with raised borders and an atrophic center. Ulceration occurs in up to 35% of cases and is notoriously difficult to treat (Elmholdt et al., 2008). This complication negatively affects quality of life and implies a greater risk for secondary infection. Although NBL is usually observed in diabetic patients, there is some controversy regarding the degree of this association and it has been hypothesized that the strength of this association may have been overestimated in the past. Some authors have studied the effect of glucose control on NBL and found no correlation with glycosylated hemoglobin A1c levels (Dandona et al., 1981), while others found an association with a poor glucose control (Cohen et al., 1996).

3.3.1 Management

There is currently no standardized effective treatment of NBL. A wide variety of treatments have been used over the years in adults. These include: topical, systemic or intra-lesional

steroids, aspirin, cyclosporin, mycophenolate, becaplermin, excision and grafting, laser surgery, hyperbaric oxygen, topical granulocytemacrophage colony-stimulating factor and photochemotherapy with topical PUVA (Hanas et al., 2009). A recent study suggests the use of TNF inhibitors in selected patients for treatment of NBL (ulcerative forms) unresponsive to prior conventional therapies (Suárez-Amor et al., 2010). NBL in children can be hard to manage and may be associated with a long-term risk of malignant transformation to squamous cell carcinoma. Systemic therapies, such as corticosteroids and azathioprine are immunosuppressive and immunomodulatory and could facilitate malignant transformation (Beattie et al., 2006). Therefore, although NBL is not clearly related to poor metabolic control, we believe that the diabetic control may also be useful. Effective primary prevention strategies and new treatment options are needed to adequately control the disease and its progression.

3.4 Osteopenia

Children and adolescents with T1D can show several impairment of bone metabolism and structure, resulting in a higher risk of decreased bone mass and its related complications later in life. Consequently an assessment of quality of the bone through non-invasive methods (phalangeal ultrasonography) seems to be opportune in the care of diabetic patients, specially the ones with clusters of autoimmune diseases to define a possible involvement of the bone (Lombardi et al.,2010).

Bone impairment in multiple autoimmune diseases might be considered not only a complication due to endocrine or nutritional mechanisms, but also a consequence of an immunoregulatory imbalance.

3.4.1 Metabolic causes

Alterations of bone mineral density (BMD) are especially observed when diabetes is associated with CD and/or AIT. Bone loss, described in patients with T1D, AIT or CD is usually viewed as a complication of these diseases and is related to duration of diabetes and quality of metabolic control. The exact mechanisms accounting for bone loss in these diseases have been variably explained by metabolic derangements due to the impaired hormonal function in T1D or AIT (McCabe, 2007), or calcium malabsorption and secondary hyperparathyroidism in untreated CD patients (Selby et al., 1999). Alterations of homeostatic mechanisms might explain an imbalance of osteoclast activity leading to osteopenia (Lombardi et al.,2010; Wu et al., 2008).

3.4.2 Immune causes

Bone remodeling involves complex interactions between osteoclasts and other cells in their microenvironment (marrow stromal cells, osteoblasts, macrophages, T-lymphocytes and marrow cells) (Kollet et al., 2007; Teitelbaum, 2007). Besides their role in calcium mobilization from bone and initiation of bone remodeling, osteoclasts are now considered as the innate immune cells in the bone, since they are able to produce and respond to cytokines and chemokines. Some authors found altered levels of plasma Osteoprotegerin (OPG) in children with T1D. Osteoprotegerin is a circulating secretory glycoprotein and is a member of the tumor necrosis factor receptor (TNFR) family. It works as a decoy receptor for the cytokine receptor activator of NFkB ligand (RANKL). RANKL and OPG are a key agonist/antagonist cytokine system: RANKL increases the pool of active osteoclasts thus

increasing bone resorption, whereas OPG, which neutralizes RANKL, has the opposite effect. Alterations or abnormalities of the RANKL/OPG system have been implicated in different metabolic bone diseases characterized by increased osteoclast differentiation and activation, and by enhanced bone resorption (Galluzzi et al., 2005). Therefore, bone could be an additional target of immune dysregulation.
Cytotoxic T lymphocyte-associated antigen-4 (CTLA4), a well-known susceptibility gene for autoimmune disorders, might also represent a possible link between immune system and bone. In animal studies CTLA4 expressed on T regulatory (Treg) cells impairs osteoclast formation (Zaiss et al., 2007). Therefore the failure of Treg cell function in clustering of multiple autoimmune diseases could represent a mechanism to explain both the occurrence of poly-reactive autoimmune processes and the increase of bone resorption in the same individuals.
In patients affected by both T1D and CD, the risk of developing osteopenia is probably influenced by the compliance to gluten-free diet. Osteopenia occurs more frequently in patients with diabetes and CD with poor compliance to GFD. Interestingly, recent observations indicate also an imbalance of cytokines relevant to bone metabolism in untreated celiac patients' sera and the direct effect of these sera on in vitro bone cell activity. In particular the RANKL/osteoprotegerin (OPG) ratio was increased in patients not on gluten-free diet. Actually, the only presence of a second disease, either AIT or CD, do not seems to increase the frequency of osteopenia, provided a good compliance to GFD in CD patients, while the association of three autoimmune diseases significantly increases the occurrence of osteopenia (37.5%). In addition, poor compliance to GFD of CD patients could increase the occurrence of osteopenia more in patients with three autoimmune diseases (80%) than in those with two autoimmune diseases (18.8%) (Valerio et al., 2008).

3.5 Gastropathy

Gastrointestinal motility disorders are found in a consistent proportion of children with T1D and are associated with significant morbidity: they are usually associated with dyspeptic symptoms, such as nausea, vomiting, fullness and epigastric discomfort, and could be an important cause of morbidity in diabetic patients. Gastroparesis has been shown to be significantly correlated with a poor metabolic control in a population of T1D children with gastric electrical abnormalities. (Cucchiara et al., 1998). Furthermore it is conceivable that delayed gastric emptying may cause a mismatch between the onset of insulin action and the delivery of nutrients into the small intestine (Rayner et al., 2001). Diabetic children with unexplained poor glycemic control should be investigated for abnormalities in gastric motility (Shen & Soffer 2000). On the other hand, hyperglycaemia itself can affect the neuromuscular mechanisms regulating gastrointestinal motility and delay the gastric emptying process (Jebbink et al., 1994). Therefore, it is of great importance to try to reverse abnormalities of gastric motility and improve gastric emptying in patients with T1D and gastroparesis by the use of domperidone in children with T1D. (Franzese et al., 2002b).

3.6 Type 1 diabetes and limited joint mobility

Type 1 diabetes can be associated with other less common disabling conditions of locomotor system: Dupuytren's contracture, stiff hand, carpal tunnel syndrome, and limited joint mobility (LJM). Limited joint mobility is one of the earliest clinically apparent long-term complications of T1D in childhood and adolescence, characterized by a bilateral painless

contracture of the finger joints and large joints, associated with tight waxy skin. Changes begin in the metacarpophalangeal and proximal interphalangeal joints of the fifth finger and extend radially with involvement of the distal interphalangeal joints as well. Involvement of larger joints includes particularly the wrist and elbow, but also ankles and cervical and thoracolumbar spine (Komatsu et al., 2004). The limitation is only mildly disabling even when severe. With rare exception, LJM appears after the age of 10 years. The prevalence of LJM in T1D, evaluated in several studies ranges from 9 to 58% in paediatric and adult patients (Lindsay et al., 2005).

The biochemical basis of LJM may be a consequence of changes in the connective tissue, probably due to alterations in the structural macromolecules of the extracellular matrix. The hyperglycaemia can alterate the glycation of protein with the formation of advanced glycation end products (AGEs), which resist to protein degradation and consequently increase thickness of basal membranes in the periarticular tissues (Shimbargger, 1987). Development of LJM is related to both age and diabetes duration (Cagliero et al., 2002), while others showed that it can be compromised also in a precocious age and with a short duration of diabetes (Komatsu et al., 2004). Of note, fluorescence of skin collagen, which reflects the accumulation of stable AGEs, increases linearly with age, but with abnormal rapidity in T1D and in correlation with the presence of retinopathy, nephropathy and neuropathy (Monnier et al., 1986).

Some authors have showed that there is a clear link between upper limb musculoskeletal abnormalities and poor metabolic control (Ramchurn et al., 2009). It has been observed a reduction in frequency of LJM between the mid-70s and mid-90s in children, most likely due to the improved glucose control during this era (Infante et al., 2001; Lindsay et al., 2005).

3.7 Type 1 diabetes and oedema

Insulin oedema is a well-recognized and extremely rare complication of insulin therapy. It was found to occur equally in both sexes in adults, but a clear female predominance was noted in younger ages. The condition is self-limiting, but a progression to overt cardiac failure and development of pleural effusion has been reported. (Chelliah & Burge, 2004).

The pathophysiology remains vague. Intensive fluid resuscitation in an insulin-deficient catabolic state may lead to extravasation of fluid to the subcutaneous tissue, resulting in peripheral oedema. This may be exacerbated by the increased capillary permeability associated with chronic hyperglycemia. Renal tubular sodium reabsorption is enhanced by insulin therapy via stimulating the Na+/K+-ATPase as well as the expression of Na+/H+ exchanger 3 in the proximal tubule. Transient inappropriate hyperaldosteronism has also been suggested to contribute to the fluid retention (Bas et al., 2010). Loss of albumin from the circulation due to increased transcapillary leakage probably contributed to the formation of oedema and the decreased serum albumin, but was not severe enough to account for the magnitude of oedema (Wheatly & Edwards 1985). Cases with normal serum albumin have also been reported.

Clinically, insulin oedema may present with a spectrum of severity until to frank anasarca. Pleural effusions have uncommonly been reported, although some of these patients were elderly and may have had pre-existing cardiac disease. Rarely, the oedema extended from peripheral tissues to serosal cavities with ascites and cardiac failure (Bas et al., 2010). Fluid and salt restriction should be implemented and this may be all that is necessary. Diuretic

therapy may be indicated in more severe decompensated cases. Administration of an aldosterone antagonist such as spironolactone may be considered from a pathophysiological point of view in the presence of inappropriate hyperaldosteronism (Kalambokis et al., 2004). In most instances no specific therapy is needed and spontaneous recovery is noted.

Impaired growth	Poor metabolic control	Monitoring of growth and physical development using growth charts
Eating disorders	Dietary restriction	Ameliorating of nutritional assistance
Necrobiosis lipoidica diabeticorum	Parallel dermopathy	Routine clinical examination of the skin
Osteopenia	Probably even present, but worsened by poor metabolic control/comorbidity	Eventually controlled by Bone ultrasonography/ DEXA
Gastropathy	Poor metabolic control	Investigating of dyspeptic symptoms
Limited joint mobility	Parallel condition	Routine clinical examination of the joint mobility
Oedema	Unknown	Clinical examination

Table 3. Non autoimmune associated conditions to Type 1 diabetes, causes and detection

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5

Hypoglycemia as a Pathological Result in Medical Praxis

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1. Introduction

Maintenance of blood glucose homeostasis is fundamentally important for health. The maintain of stable levels of glucose in the blood is one of the most finely regulated of all homeostatic mechanisms and one in which the liver, the extrahepatic tissues, and several hormones play a part. Even mild disruptions of glucose homeostasis can have adverse consequences.

The physiological post absorptive serum glucose concentration in healthy humans range is 4, 4-5,8 mmol/L (80 to 105 mg/dL). The stability of the plasma glucose level is a reflection of the balance between the rates of whole body glucose production and glucose utilization.

Generally, hypoglycemia is defined as a serum glucose level below 3. 8 mmol/L (70 mg/dL).As a relatively rare disorder, hypoglycemia most often affects those humans at the extremes of age, such as infants and the elderly, but may happen at any age.

As a medical problem, hypoglycemia is diagnosed by the presence of three key features (known as Whipple's triad). Whipple's triad is:

1. symptoms consistent with hypoglycemia,
2. a low plasma glucose concentration, and
3. relief of symptoms after the plasma glucose level is raised.

The etiology of hypoglycemia is numerous:

1. Inborn error of metabolism (more common in the pediatric patient than in adults). Disturbance in carbohydrates metabolism: Malabsorption of glucose/galactose, alactasia, asucrasia, galactosemia, fructose intolerance. Glycogen storage disease, Type I ,or von Gierke Disease, glycogen storage disease, Type III, a deficiency of glycogen disbranching enzyme activity (limit dextrinosis), Type VI glycogen storage disease, a deficiency of liver phosphorylase)
2. **Hormonal disturbance** .The hormone insulin plays a central role in the regulation of the blood glucose concentration. It is produced by the β cells of islets of Langerhance in the pancreas. Insulin exerts hypoglycemic effect. Glucagon is the hormone produced by the α

cells of Langerhance islets in the pancreas. Its secretion is stimulated by hypoglycemia. The hormone glucagon, epinephrine, norepinephrine, growth hormone, and cortisol exert the opposite effects to insulin and they belong in the counter-regulatory hormones

3. The disorders of some organs (Liver and Kidney Disorders especially). Any abnormality in the functioning of the liver can disturb the process of blood-sugar regulation, resulting in hypoglycemia. On the other hand, kidney disorder can be one of the major causes of low blood sugar.
4. Infection-related hypoglycemia (in older adults)
5. The adverse medication reactions
6. Hypoglycemia as the complication of treatment of diabetes mellitus.

The central nervous system requires glucose as its primary fuel. The brain uses more than 30% of blood glucose. The brain does not produce the glucose required for its functioning and it is completely dependent on the rest of the body for its supply. So, fluctuations in blood sugar levels can prove to be harmful for the brain; a continual supply of glucose is necessary as a source of energy for the nervous system and some other organs like erythrocytes, testes and kidney medulla .Gluconeogenesis, the biosynthesis of new glucose, (i.e. not glucose from glycogen) from other metabolites (lactic acid, amino acids and glycerol) is necessary for use as a fuel, since glucose is the sole energy source for these organs.

The symptoms caused by low blood sugar come from two sources and may resemble other medical conditions. The first symptoms are caused by the release of epinephrine from the nervous system. These include sweating, pale skin color, shakiness, trembling, rapid heart rate, a feeling of anxiety, nervousness, weakness, hunger, nausea and vomiting. Lowering of the brain's glucose causes: headache, difficulty in thinking, changes in vision, lethargy, restlessness, inability to concentrate or pay attention, mental confusion, sleepiness, stupor, and personality changes.

To treat low blood sugar immediately the patients should eat or drink something that has sugar in it, such as orange juice, milk, or a hard candy. It is need to find out the causes of hypoglycemia.

Laboratory diagnosis of hypoglycemia is very important in medical praxis especially in pediatric, internal medicine (hepatology, renal failure, and cardiology) neuropsychiatry disorders and so on.

Glucose is the name of the simple sugar found in plant and animal tissues. It is made within plants as a product of photosynthesis. Although glucose can be produced within the human body, most of it is supplied to people by dietary carbohydrate intake principally as starch. Once consumed and digested, glucose will either be used immediately or stored as glycogen for future use, (Caraway & Watts,1986; Mayer,1975).

Glucose is the major energy source for human body and is derived primarily from dietary carbohydrates (grains, starchy vegetables, and legumes), from body stores of carbohydrates (glycogen) and from the synthesis of glucose from protein and glycerol moiety of triglycerides (gluconeogenesis) (King, 2011).

The glucose level in blood is kept within narrow range through a variety of influences. While there is some variation in blood glucose as circumstance changes (feeding, prolonged fasting), levels above or below the normal range usually indicate disease.

High blood glucose due to diabetes mellitus is the most commonly encountered disorder of carbohydrate metabolism. Low blood glucose is an uncommon cause of serious diseases. There are numerous rare conditions that cause hypoglycemia in neonatal period and early

childhood. In adults, low blood glucose in the fasting state is almost always due to a serious underlying condition (Caraway & Watts,1986; King, 2011; Mayer, 1975; Service,1992).

2. Digestion and absorption of carbohydrates

Carbohydrates are important components of the diet. The carbohydrates that we ingest range from simple monosaccharides (glucose, fructose and galactose) to disaccharides (lactose, sucrose) and complex polysaccharides, starch and glycogen. Most carbohydrates are digested by salivary and pancreatic amylases, and are further broken down into monosaccharides by enzymes in the brush border membrane (BBM) of enterocytes. Maltase, lactase and sucrase-isomaltase are disaccharidases involved in the hydrolysis of nutritionally important disaccharides, maltose, lactose, saccharose. Once monosaccharides are presented to the BBM, mature enterocytes, expressing nutrient transporters, transport the sugars into the enterocytes, (Drozdowski & Thomson, 2006).

The resultant glucose and other simple carbohydrates, galactose (from lactose) and fructose (from succrose) are transported across the intestinal wall to the hepatic portal vein and then to liver parenchymal cells. Both fructose and galactose are readily converted to glucose by hepatocytes Absorption of glucose and galactose occurs by an active carrier-mediated transfer process. Fructose is absorbed by facilitated diffusion (Harper,1975; King, 2011; www.deo.ucsf.edu/type1/understanding-diabetes).

Fructose and galactose are phosphorylated by specific enzymes, fructokinase and galactokinase, presented only in the liver, and converted to glucose. Glucose is transported from the liver via the bloodstream to be used by all the body cells as the most important source of energy. Glucose, as unique sugar in systemic blood circulation, leaves the blood, enters cells through specific transport proteins and has one principal fate: it is phosphorylated by ATP to form glucose-6-phosphate by hexokinase in all human body cells or by the action of glucokinase in hepatocytes. This step is notable because glucose-6-phosphate cannot diffuse through the membrane out of the cells (Haris, 1997; Harper,1979; King,2011; Tietz,1986; Voet & Voet, 2004a).

3. Glycemia - Physiological regulation

Maintenance of blood glucose homeostasis is fundamentally important for health. The plasma glucose level is tightly controlled throughout life in the normal individual, in spite of intermittent food ingestion and periods of fasting, as the net balance between the rates of glucose production and utilization. The stability of the plasma glucose level is a reflection of the balance between the rates of whole body glucose production and glucose utilization.

The amount of plasma glucose level in healthy humans is usually maintained within a range of 4.4 to 5.8 mmol/L , 80 to 110 mg/dL), (Carraway & Watts,1986; King, 2011; Mayes,1975; Service, 1992; Voet & Voet, 2004a).

4. Intermediary metabolism of carbohydrates

4.1 Glycogen synthesis

Glycogen is the storage form of glucose and serves as a tissue reserve for the body's glucose needs. Glycogen synthesis occurs in virtually all animal tissues, but it is especially prominent in the liver and skeletal muscles. In the liver, glycogen serves as a reservoir of

glucose, readily converted into blood glucose for distribution to other tissues, whereas in muscles glycogen is broken down via glycolysis to provide energy for muscle contraction. In human body, glycogen is synthesized and stored when glucose levels are high and is broken down during starvation or periods of high glucose demand.

Glycogen is a highly branched polymeric structure containing glucose as the basic monomer (Mayes, 1975; Voet & Voet, 2004b). It is composed of polymers of α-1-4 linked glucose, interrupted by α 1-6 linked branch point every 4-10 residues (Fig 1).

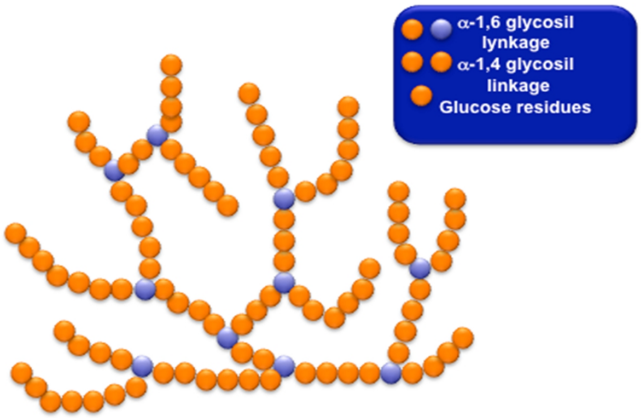

Fig. 1. Glycogen structure

Uridine diphosphate glucose (UDP-glucose) is the immediate precursor for glycogen synthesis. Glycogen synthase will only add glucose units from UDP-glucose onto a preexisting glycogen chain that has at least four glucose residues. Linkage of the first few glucose units to form the minimal "primer" needed for glycogen synthase recognition is catalyzed by a protein called glycogenin, which attaches to the first glucose and catalyzes linkage of the first eight glucoses by alpha(1,4) bonds. The enzyme, glycogenin, initiates glycogenynthesis (oregonstate.edu/.../summer09/lecture/glycogennotes.html; Voet & Voet, 2004b).

The enzyme glycogen synthase then catalyzes elongation of glycogen chains initiated by glycogenin to a chane of 9 – 11 glucose molecule. Glycogen synthase catalyzes transfer of the glucose moiety of UDP-glucose to the hydroxyl at C_4 of the terminal residue of a glycogen chain to form an α(1-4)-glycosidic linkage (Fig 2) (Mayes,1975; King, 2011; Voet & Voet, 2004b; www.uic.edu/.../glycogen%20metab/Glycogen%20biochemistry.htm).

A branching enzyme forms the branching points in glycogen. The branches arise from α-(1-6) linkages which occur every 8 to 12 residues. Glycogen branches are formed by amylo-(1,4-1,6)-transglycosylase, also known as branching enzyme. The branching enzyme transfers a segment from the end of a glycogen chain to the C6 hydroxyl of a glucose residue of glycogen to yield a branch with an α-(1-6) linkage. In the presence of glycogenin, glycogen synthase, branching enzyme and UDP glucose (active glucose) form glycogen as a highly branched polymeric structure, containing glucose as the basic monomer (Figure 2).

Glycogen is synthesized and stored mainly in the liver and the muscles as well as in the cytoplasm of all human body cells as granules named ”residual bodies", (Voet & Voet, 2004b).

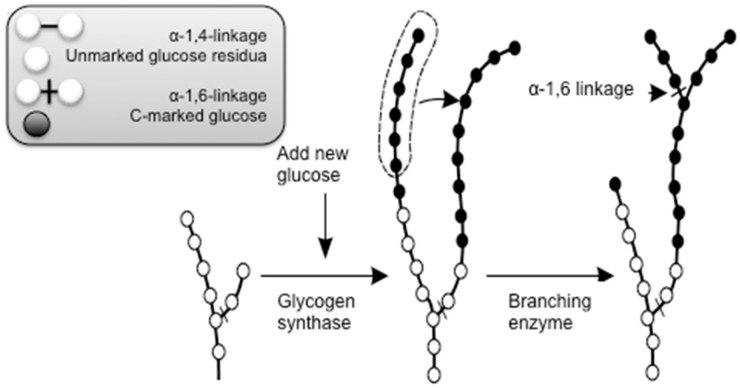

Fig. 2. Glycogenesis

4.2 Glycogen breakdown (glycogenolysis)

In the proceses of glycogen catabolism or glycogenolysis, glycogen, stored in the liver and muscles, is converted first to glucose-1-phosphate and then into glucose-6-phosphate. (Mayes,1975; Voet &;Voet, 2004b). Three enzymes participate in glycogenolysis: glycogen phosphorylase, oligo-1,4-1,4–glucan transferase or trisaccharide transferase, and α(1-6) glucosidase or γ- amylase. Glycogen phosphorylase catalyzes phosphorolytic cleavage of the α-1,4 glycosidic linkages of glycogen (using inorganic phosphate), releasing glucose-1-phosphate as reaction product and limit dextrin. After extensive phosphorylase action on glycogen, the molecule contains four glucose residues in α-1,4-glucosidic bond attached by a(1,6)-link to the glycogen molecule.

These structures can be further degraded by the action of a debranching enzyme, which carries out two distinct reactions. The first of these, known as oligo-a1,4-a-1,4) glucan transferase activity or trisaccharide transferase, removes a trisaccharide unit from limit branch and transfers this group to the end of another nearby glycogen chain, with resynthesis of the α-1,4 bond. This leaves a single glucose residue in a-(1,6) linkage to the main chain. The α-1,6-glucosidase or γ-amylase activity of the debranching enzyme then catalyzes hydrolysis of the α(1,6) linkage, leaving a polysaccharide chain with one branch fewer and yielding free glucose. This is a minor fraction of free glucose released from glycogen (Fig 3), since that the major product of glycogen breakdown by phosphorylase activity is glucose-1-phosphate. Phosphoglucomutase catalyzes the reaction: glucose-1-phosphate → glucose-6-phosphate.

Glucose-6-phosphate is the first step of the glycolysis pathway if glycogen is the carbohydrate source of further energy needed. If energy is not immediately needed, the glucose-6-phosphate is converted to glucose, by the action of the enzyme glucose-6-phosphatase (mainly in liver), for distribution to various cells by blood, such as brain, erythrocytes, adipocytes, etc.

The reactions involved in tissue glycogen synthesis and degradation are carefully controlled and regulated by hormones. The primary hormone responsible for conversion of glucose to glycogen is insulin. Opposite effects to glycogen metabolism have its antagonists: glucagon, adrenaline, cortisol, growth hormone which facilitate glycogenolysis in liver and muscles.

The principal enzymes of glycogen metabolism are glycogen synthase and glycogen phosphorylase, reciprocally regulated by allosteric effectors and covalent modification (through phosphorylation or dephosphorylation). Glycogen synthase is active when high blood glucose leads to intracellular glucose-6-P increase. Glycogen phosphorylase is active in the presence of high level of cyclic adenosine monophosphate (cAMP) which suggests that the cells need chemical energy in the form of ATP.

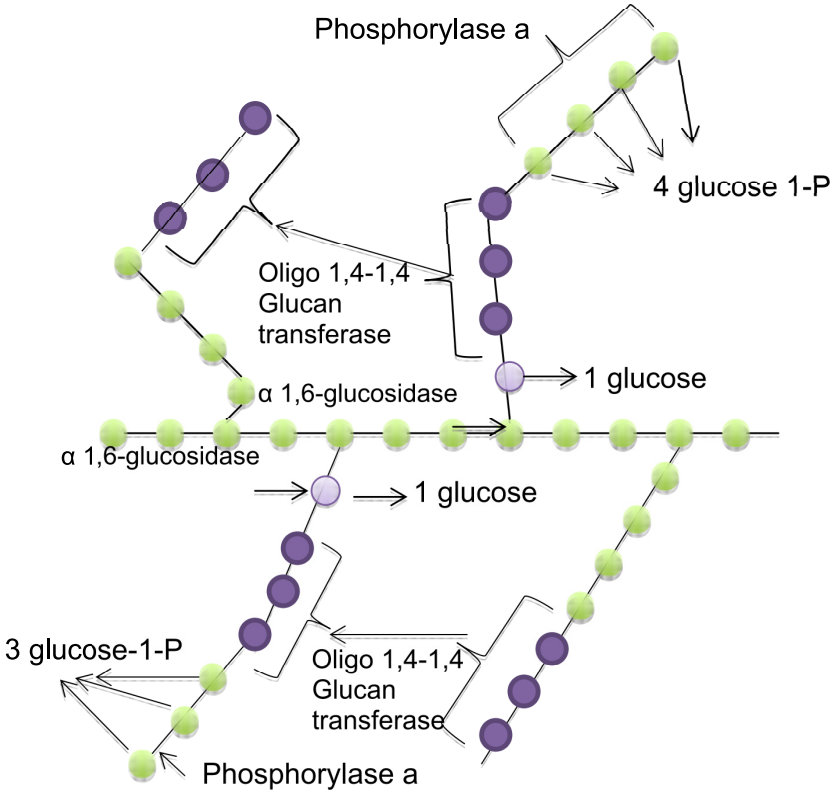

Fig. 3. Glycogenolysis

Glucagon, synthesized by pancreatic α-cells, and epinephrine (adrenaline), synthesized by adrenal medulla, regulate glycogen metabolism by covalent modification (phosphorylation and dephosphorylation) through cAMP cascades. Both hormones are produced in response to low blood glucose level. Glucagon activates cAMP formation in liver, while adrenaline activates its formation in muscle. Phosphorylation of the enzyme, via cAMP cascade, induced by adrenaline, results in further activation of glycogen phosphorylase. These regulatory processes ensure release of phosphorylated glucose from glycogen, for entry into glycolysis to provide ATP needed for muscle contraction.

Insulin, produced in response to high blood glucose, antagonizes effects of the cAMP cascade induced by glucagon and adrenaline. It is the only hormone inducing cAMP decrease (Mayes, 1975; Voet & Voet, 2004b).

4.3 Glycolysis

ATP depletion in cells, or low blood glucose level, lead to the activation of glycogenolysis and the enhancement of glucose degradation through glycolysis. Glycolysis is a central metabolic pathway of glucose metabolism, starting with glucose-6-phosphate, produced by glycogenolysis or gluconeogenesis. Glucose-6-phosphate could also be synthesized directly from blood-derived glucose by the action of hexokinase in all human body cells or by the action of glucokinase in hepatocytes.

Glycolysis is the anaerobic catabolism of glucose. It occurs in cytosol of virtually all cells. The glycolytic pathway converts a molecule of glucose into 2 molecules of pyruvic acid and captures 2 molecules of ATP. If glycolysis proceeds in aerobic conditions 2 molecules of pyruvic acid enter mitochondria, transforms into acetyl-CoA which is oxidized by the citric acid cycle. One cycle provides 12 mol ATP per one molecule of pyruvate. Aerobic conditions provide a mechanism for converting NADH back to NAD^+ which is essential for glycolysis to operate (Fig 4).

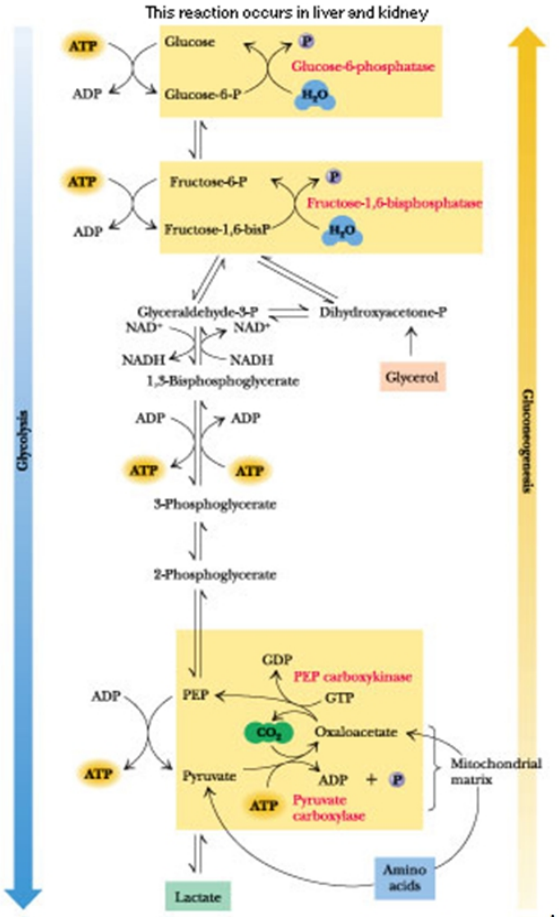

Fig. 4. Glycolysis and Gluconeogenesis

Under anaerobic conditions 2 molecules of pyruvate, under the action of lactate dehydrogenaze, and by using $NADH_2$, convert to 2 molecules of lactate. The reaction is freely reversible (Haris, 1997; Mayes, 1975; Voet & Voet, 2004b; users.rcn.com/.../I/ IntermediaryMetabolism.html).

4.4 Gluconeogenesis

If glucose is not obtained in the diet, during fasting , the body must produce new glucose from noncarbohydrate precursors by the proces of gluconeogenesis. The term gluconeogenesis means the generation (*genesis*) of new (*neo*) glucose.

The production of glucose from other metabolites is necessary to maintain the glucose level in the blood as a fuel source by the brain, erythrocytes, kidney medulla and testes, since glucose is the sole energy source for these organs. During starvation, however, the brain can derive energy from ketone bodies which are converted to acetyl-CoA. The adipose tissue needs glucose which is also necessary for the synthesis of triacylglycerls and glycerophospholipids. The main precursors for gluconeogenesis are lactate and alanine from muscle, glycerol from adipose tissue, and glucogenic amino acids from the proteolysis in peripheral tissues and proteins from the diet. The most of the amino acids, as well as their α-keto acids, are TCA cycle intermediates. In addition, the gluconeogenetic processes are used to clear the intermediary products of metabolism of other tissues from the blood, e.g. lactate, produced by muscles and erythrocytes, and glycerol, which is continuously produced by adipose tissue.The principal organs responsible for gluconeogenesis are the liver and kidneys, which account for about 90% and 10% of the body's gluconeogenic activity, respectively. Interestingly, the mammalian organs that consume the most glucose, namely, brain and muscle, carry out very little glucose synthesis (Gerich et al, 2001; King, 2011; Mayes,1975; Voet & Voet, 2004b; Woerle & Stumvoll, 2001).

Gluconeogenesis is similar but not the exact reverse of glycolysis; some of the steps are the identical in reverse direction and three of them are new ones (Fig 4). In glycolysis energy barriers obstruct a simple reversal of glycolysis: reactions catalyzed by pyruvate kinase, phospho-fructokinase and hexokinase. These barriers are circumvented by new, special enzymes of gluconeogenesis: pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructoso-1,6-diphosphatase and glucose-6-phosphatase. The conversion of lactate to glucose begins with the oxidation of lactate, by the action of lactate dehydrogenase, to pyruvate. In the presence of ATP, pyruvate carboxylase and CO_2 convert pyruvat to oxaloacetate. The enzyme, phosphoenolpyruvate carboxykinase (PEPCK) transfers oxaloacetate to phosphoenolpyruvate in the presence of GTP and by elimination of CO_2. Thus, with the help of these two enzymes, and lactate dehydrogenase, lactate can be converted to oxaloacetate. The pyruvate and oxaloacetate are the intermediary products of catabolic pathway of many glycolytic amino acids. The next steps of reversal glycolysis continue just to formation of fructose-1,6-diphosphate, the substrate for fructose-1, 6-diphosphatase. Fructose-6-phosphate, formed by elimination of inorganic phosphate, converts to glucose-6-phosphate (G6P). The energy required for the hepatic synthesis of glucose from lactate is derived from the oxidation of fatty acids. In the liver and kidney, G6P can be dephosphorylated to glucose by the enzyme glucose 6-phosphatase. This is the final step in the gluconeogenesis pathway.

4.4.1 Cory cycle

Lactate, formed by the oxidation of glucose in skeletal muscles and by erythrocytes through the proceses of anaerobic glycolysis, is transported to the liver and kidney, where it re-forms glucose, which again become available via the circulation for oxidation in the tissues. This process is known as the Cory cycle or lactic acid cycle (Fig 5).

4.4.2 Glucose-alanine cycle

It has been noted that of the amino acids transported from muscles to the liver during starvation or under the action of cortisol, alanine predominate. Glucose-alanine cycle represents a cycling glucose from the liver to the muscles and alanine from muscles to liver, effecting a net transfer of amino nitrogen from muscle to liver and free energy from liver to muscle. At the level of muscles, pyruvate, formed by glycolysis, transforms to alanine by the action of alanine transaminase (ALT) or glutamate pyruvat transaminase (GPT). The reaction is freely reversible; at the level of hepatocytes alanine transfers to pyruvate by the action of the same anzyme (Fig 5).

Glycerol, necessary for the synthesis of triacylglicerols and glycerophosholipids is derived, initially, from the blood glucose since free glycerol cannot be utilised readily for the synthesis of these lipids in tissues. Instead of free glycerol, adipose tissue uses α-glycero phosphate or ”active glycerol" produced during degradation of glucose by glycolysis.

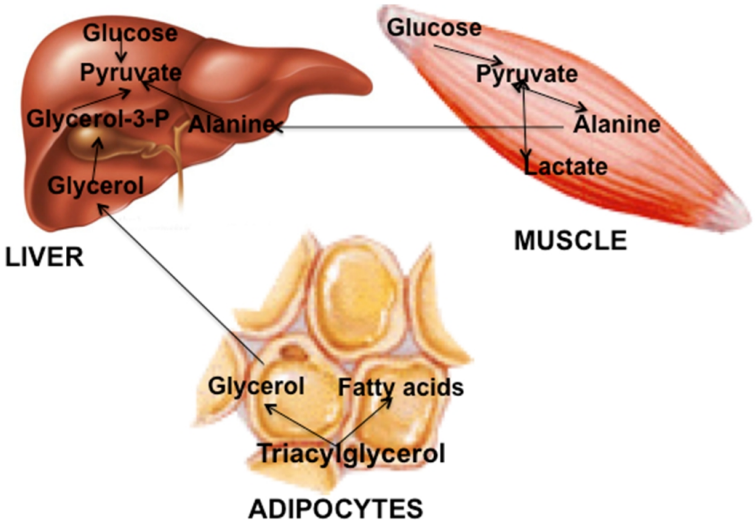

Fig. 5. Cory cycle , glucose-alanine cycle and glycerol-glucose cycle

4.4.3 Glycerol - Glucose cycle

Glycerol, a product of the continual lipolysis, diffuses out of the tissue into the blood. It is converted back to glucose by gluconeogenic mechanisms in the liver and kidney. Thus, a continous cycle exists in which glucose is transported from the liver to adipose tissue and, hence, glycerol is returned to be synthesized into glucose by the liver. Glycerokinase, which requires ATP, catalyzes the conversion of glycerol to α-glycero phosphate. Glycerokinase is present in liver and kidney. The enzyme α -glycero phosphate dehydrogenase oxidizes α-glycero phosphate to the dihydroxacetone phosphate, the component of glycolysis, which enters the glycolytic pathway as a substrate for triose phosphate isomerase (Fig 4). Thus, liver is able to convert glycerol to blood glucose by making use of above enzymes - some of the enzyme of glycolysis and specific enzymes of gluconeogenic pathway, fructose-1,6-diphosphatase and glucose-6-phosphatase(Harris & Crabb, 1997; King, 2011; Mayes,1975).
Glucose produced by gluconeogenesis in the liver and kidney is released into the blood and is subsequently absorbed by all human body cells especially brain, heart, muscle, and red blood cells to meet their metabolic needs. In turn, pyruvate, lactate and glycerol produced in these tissues are returned to liver and kidney to be used as gluconeogenic substrates.

5. The physiological regulation of carbohydrate metabolism

5.1 Glucose homeostasis

The maintaining of stable levels of blood glucose is one of the most finely regulated of all homeostatic mechanisms and one in which the liver, extrahepatic tissues and several hormones play a part. Even mild disruptions of glucose homeostasis can have adverse consequences. The physiological post absorptive serum glucose concentration in healthy humans range is 4. 4-5.8 mmol/L (80 to 110 mg/dL).
Glycemia is controlled by several physiological processes. It tends to fluctuate to higher levels after meals, due to intestinal absorption of carbohydrates of low molecular weight present in the diet or broken down polysaccharides, such as starch or glycogen. On the other hand, glucose tends to decrease to lower levels induced by cell metabolism, particularly after stress, temperature regulation and physical exercise. Glucose can also be supplied via breakdown of cellular reserves of glycogen. Another input to glycemia levels is gluconeogenesis, whereby glycogen stored in the liver and skeletal muscles are depleted.
The stability of the glycemia is a reflection of the balance between the rates of whole body glucose production and glucose utilization. The glucose homeostasis is tightly regulated by the levels of hormones and substrates in blood and by the physiologic functions of body tissues and organs (Carraway & Watts, 1986; Haris, 1997; King,2011).

5.2 Hormonal regulation of glycemia

The hormones involved in glycemia regulation include insulin (which lowers the blood sugar level) and other hormons which raise blood sugar, namely antagonists of insulin such as glucagon, epinephrine, cortisol, growth hormone, thyreoid hormones (T3 and T4) and many others. The proper functions of these hormones is precise control of glucose concentration in the blood. Insulin and glucagon are two major hormones involved in regulation of blood glucose level. They are both secreted in response to blood sugar levels, but in opposite fashion. At the same time, enhanced insulin secretion induced increased glucagon secretion.

Insulin has a hypoglycemic effect. Secretion of insulin is a response to increased glucose level in the blood. In addition to the direct effects of hyperglycemia in enhancing the uptake of glucose into both the liver and peripheral tissues, the hormon insulin plays a central role in the regulation of the blood glucose concentration. Similarly, as blood glucose falls, the amount of insulin secreted by the pancreatic islets goes down.
Glucagon, as a direct antagonist of insulin, has a hyperglycemic effect. Secretion of glucagon is a response to decreased glucose level in the blood (Chattoraj & Watts,1986; Ginsberg, 1990 a, 1990b; Mayes,1975; King, 2011).

5.2.1 Insulin

Insulin is a small protein consisting of an alpha chain of 21 amino acids linked by two disulfide (S—S) bridges to a beta chain of 30 amino acids. The precursor of insulin is a proinsulin, which contains C peptide (conective peptide). The conversion of proinsulin to insulin requires biologic proteolysis (Ginsberg,1990; Bowen, 2010; Harper, 1975; Chattoraj & Watts,1986).
The stimulus for insulin secretion is a high blood glucose. Insulin is produced by β cells of Langerhans islets in pancreas and is secreted into the blood as a direct response to hyperglycemuia. Beta cells have channels in their plasma membrane that serve as glucose detectors. When blood glucose levels rise (after a meal), insulin is secreted from the pancreas into the pancreatic vein, which empties into the portal vein system, so that insulin traverses the liver before it enters the systemic blood supply. Insulin acts to rapidly lower blood glucose concentration in several ways. It stimulates the active transport of glucose across plasma membranes through glucose transporter (GLUT 4) of muscle and adipose tissue. The liver, brain and red blood cells do not require insulin for glucose uptake. Insulin is an anabolic hormone. It promotes anabolic processes in these cells, such as increasing the rate of glycogenesis, lipidogenesis and proteins synthesis. The cellular uptake of glucose from the blood have the net effect of lowering the high blood glucose levels into the normal range. Insulin stimulates cells in most tissues of the body to preferentially use glucose as their metabolic fuel. It increases cellular glucose utilization by inducing the synthesis of several important glycolytic enzymes, namely, hexokinase, glucokinase, phosphofructokinase, and pyruvate kinase. In addition, insulin inhibit gluconeogenesis in liver. All of these physiological effects of insulin serve to lower blood glucose levels. In each case, insulin triggers these effects by binding to the insulin receptor - a transmembrane protein embedded in the plasma membrane of the responding cells. When the the glucose concentration in the blood falls, pancreas stops releasing insulin (Ginsberg, 1990 a, 1990b; Bowen, 2007; King, 2011).

5.2.2 Amylin

Pancreatic beta cells secrete amylin, a peptide of 37 amino acids. All of its actions (inhibition of glucagon secretion, slowing down the stomach emptying, sending a satiety signal to the brain) tend to supplement those of insulin, reducing the level of glucose in the blood (King, 2011; Silvestre et al, 2001; Young, 2005; users.rcn.com/.../I/IntermediaryMetabolism.html).

5.2.3 Glucagon

Glucagon is another 29 amino acid peptide hormone produced by pancreas. It is a hyperglycemic hormone (Bowen, 2007). Glucagon is produced by alpha (α) cells of

Langerhans islets as proglucagon and proteolytically processed to yield glucagon within alpha cells of the pancreatic islets. Proglucagon is also expressed within the intestinal tract, where it is processed not into glucagon, but to a family of glucagon-like peptides (GLP) (Ginsberg,1990b; Bowen, 2007). Glucagon is secreted in response to hypoglycemia. It is active in liver and adipose tissue, but not in other tissues. This peptide hormone travels through the blood to specific receptors on hepatocytes and adipocytes. When the concentration of glucose in blood decreases, α cells of the pancreas begin to release glucagon. Glucagon stimulates hepatocytes to glycogenolysis and gluconeogenesis, resulting in hyperglycemia. It increases the amount of cAMP and stimulates lipolysis, contributing to reduction of the cellular glucose utilization, the increasing of lipolysis in adipose tissue, providing glycerol and free fatty acids which enter β oxidation cycle, producing the chemical energy (ATP) to most cells. Glycerol leaves the adipose tissue, and through the blood enters the hepatocytes where it may serve as the substrate in the process of gluconeogenesis (Fig 5).

5.2.4 Epinephrine (adrenaline)

Epinephrine is a hormone of adrenal medulla, which consists of masses of neurons that are the part of the sympathetic branch of the autonomic nervous system. Instead of releasing their neurotransmitters at a synapse, these neurons release them into the blood. Thus, although part of the nervous system, the adrenal medulla functions as an endocrine gland. It releases catecholamines: adrenaline (epinephrine) and noradrenalin (also called norepinephrine).

Synthesis of both catecholamines begins with the amino acid tyrosine, which is taken up by chromaffin cells. Called the "fight or flight" hormone, adrenaline prepares the organism for mobilization of large amounts of energy and dealing with stress. Together with cortisol and growth hormone they are named "stress hormones". Following release into blood, these hormones bind adrenergic receptors on target cells, where they induce essentially the same effects as direct sympathetic nervous stimulation. Adrenaline acts on liver and muscles. Mechanisms of the actions of adrenaline are the same as the mechanisms of glucagon. Through augmentation of cAMP in the cells, adrenaline initiates the enzyme cascade which leads to the activation of glycogen phosphorylase, leading to rapid breakdown of glycogen, inhibition of glycogen synthesis, stimulation of glycolysis and production of energy. In fat cell, it stimulates lipolysis, providing fatty acids as energy source in many tissues. Stimulation of lipolysis contributes to the reduction of the cellular glucose utilization and aids in conservation of dwindling reserves of blood glucose. The stimulation of hepatocytes to glycogenolysis and gluconeogenesis results in regulation of glycemia (Ginsberg,1990a; Chattoraj, & Watts, 1986; www.ncbi.nlm.nih.gov/books/NBK22429/).

5.2.5 Glucocorticoids

The glucocorticoids (cortisol as the principal one) get their name from their effect of raising the level of blood glucose. Glucocorticoids are a class of steroid hormones, synthesized and secreted from zone fasciculate of adrenal cortex, that exert distinct effects on liver, skeletal muscles, and adipose tissue (Bjelakovic et al,2008,2009). The effects of cortisol are best described as catabolic, because it promotes protein breakdown and decreases protein synthesis in skeletal muscles. However, in the liver, it stimulates gluconeogenesis, inducing increased gene expression of several enzymes of the gluconeogenic pathway. Cortisol-

induced gluconeogenesis results, primarily, in increased conversion of glycogenic amino acids (from protein breakdown in peripheral tissues) and glycerol (from fat) into glucose (Ginsberg, 1992c; Chattoraj & Watts, 1986; Gil, 1992; Litwak & Schmidt, 1997a; users.rcn.com/.../I/IntermediaryMetabolism.html.)

5.2.6 Growth hormone (GH)

Human growth hormone (GH; also called somatotropin), the protein of 191 amino acids, secreted by somatotrophs of the anterior part of pytuatary gland, regulates overall body and cell growth, carbohydrate, protein and lipid metabolism, and water-electrolyte balance. The GH-secreting cells are stimulated by growth hormone releasing hormone (GHRH) from hypothalamus and inhibited by somatostatin. The release of GH might be regulated not only by hypothalamic GHRH, but also by ghrelin derived from the stomach (Kojima et al., 2005). GH promotes body growth by binding to receptors on the surface of liver cells and stimulates them to release insulin-like growth factor-1 (IGF-1, also known as somatomedin). GH exerts the hyperglycemic effect, stimulating glycogenolysis and lypolysis in peripheral tissues. In liver, GH also stimulates glycogenolysis and glyconeogenesis (Barry, 1992c; Frohman,1992; Litwak.& Schmidt, 1997b).

5.2.7 Thyroid hormones

Thyroid hormones are derivatives of the amino acid tyrosine bound covalently to iodine. The two principal thyroid hormones are: triiodothyronin (T3) and thyroxin (T4). Thyroid hormones receptors are intracellular DNA-binding proteins that function as hormone-responsive transcription factors. The effect of the hormone-receptor complex binding to DNA is to modulate gene expression, either by stimulating or inhibiting transcription of specific genes. It is likely that all cells in the body are targets for thyroid hormones. Thyroid hormones affect oxidative metabolism, especially the metabolism of carbohydrates. Thyroid hormones enhance glucose absorption and the utilization of carbohydrates. They stimulate both the synthesis and disposal of glucose Hypothyroidism or thyroid hormone deficiency leads to decrease in basal metabolic rate and hypoglycemia (Bowen, 2010; Harper, 1975; Zmire et al, 1999).

6. Physiological functions of liver, kidneys and brain in carbohydrate metabolism

Beside hormones, some organs have the important roles in glycemia regulation. Among them, the most important are liver, kidneys and brain.

6.1 The role of liver in carbohydrate metabolism

The metabolic activities of the liver are essential for providing fuel to the brain, muscle, and other peripheral organs. The liver removes two-thirds of the glucose from the blood and all of the remaining monosaccharides (Lehninger,1977; Cherrington,1999). The absorbed glucose is converted into glucose 6-phosphate by hexokinase and the liver-specific glucokinase, whose K_m (Michaelis constant) for glucose is sufficiently higher than the normal circulating concentration of glucose (5mM). The liver plays a unique role in controlling carbohydrate metabolism by maintaining glucose concentrations in a normal range. It possesses the key enzymes for glucose intake (hexokinase and glucokinase) and for

releasing of glucose from hepatocytes (glucose-6-phosphatase). The liver has a great capability for synthesis and storing of glycogen (glycogenesis), and, in opposite direction, for glycogen breakdown (glycogenolysis). Also, the liver is the place for gluconeogenesis. (King.2011, Nordlie et al, 1999; Radziuk & Pye, 2001).
Glucose-6-phosphatase liberates free glucose molecules from hepatocytes into blood, catalyzing the following reaction: glucose-6-phosphate + H2O $\rightarrow$ glucose + Pi.The substrate for this enzyme is glucose-6-phosphate, the product of glycogenolysis or the end product of gluconeogenesis (Berg, 2002; Radziuk & Pye, 2001; Raddatz & Ramadori, 2007; Yamashita et al, 2001; Berg, 2002) .

6.2 The role of kidney in carbohydrate metabolism

Kidney may play a significant role in carbohydrate metabolism under both physiological and pathological conditions due to renal gluconeogenesis (King, 2011; Gerich et al., 2001). Although the liver is the major site of glucose homeostasis, the kidney plays a vital role in the overall process of regulating the level of blood glucose. Glucose is continually filtered by the glomeruli but is ordinarily returned completely to the blood by the enzymatic reabsorptive system of the renal tubules. The reabsorption of glucose is a process which is similar to that responsible for the absorption of this sugar from the intestine. The capacity of tubular system to reabsorb glucose is limited by the capacity of enzymatically systems of the tubule cells to a rate of about 350 mg/ minute, representing as tubular maximum for glucose (Tmg). Due to that capacity of the kidney, the definitive urine doesn't contain glucose. When the blood levels of glucose are elevated, the glomerular filtrate may contain more glucose then can be reabsorbed; the excess passes into urine to produce glycosuria. In normal individuals, glucosuria occures when the venous blood sugar excesss 9.5-10 mmol/L (170-180 mg/100 ml). This level of the venous blood sugar is termed the renal threshold for glucose (Mayes,1975; Woerle & Stumvoll, 2001).

6.3 The role of brain in carbohydrate metabolism

Glucose is the major energy source for maintenance of brain metabolism and function, except during prolonged starvation. However, the brain has limited glucose reserves and needs a continuous supply of glucose. Endogenous glucose provides more than 90% of energy needed for brain function (Cryer, 1997; Gerich et al, 2001; Halmos & Suba,2011; King, 2011). Since the brain cannot synthesize glucose or store more than a few minutes' supply as glycogen, it is critically dependent on a continuous supply of glucose from the circulation. Fatty acids do not serve as fuel for the brain, because they are bound to albumins in plasma and so do not traverse the blood-brain barrier. In prolonged starvation, ketone bodies, generated by the liver, partly replace glucose as fuel for the brain, (Cahill, 2006).
Glucose is transported into brain cells by the glucose transporter GLUT3. This transporter has a low KM for glucose (1.6 mM). Thus, the brain is usually provided with a constant supply of glucose. At normal (or elevated) arterial glucose concentrations, the rate of blood-to-brain glucose transport exceeds the rate of brain glucose metabolism. However, as arterial glucose levels fall below the physiological range, blood-to-brain glucose transport becomes limiting to brain glucose metabolism, and ultimately survival.
Nowadays it is hypothesised that the brain, in particular the hypothalamus, has a great role in carbohydrate metabolism and glucose homeostasis. The brain is an insulin-sensitive organ. Brain-insulin action is required for intact glucose homeostasis. Receptors for insulin

are concentrated in hypothalamic area. Hypothalamus is the site of afferent and efferent stimuli between special nuclei and β- cells and α cells of pancreas, and it regulates induction/inhibition of glucose output from the liver. Insulin gets across the blood-brain barrier, links to special hypothalamic receptors, regulating peripheral glucose (the hypothalamus-pancreas) (Halmos & Suba, 2011). In addition, the hypothalamus can affect metabolic functions by neuroendocrine connections: the hypothalamus-pancreas axis (the control of insulin and glucagone release), the hypothalamus-adrenal axis (the control of the release of adrenaline and noradrenaline) and the hypothalamus-pituitary axis (release of glucocorticoids and thyroid hormones through adrenocorticotrophic hormone (ACTH) and thyroid-stimulating hormone (TSH) control, respectively, which modulate glucose metabolism.

Recently, evidence is accumulating demonstrating that gastrointestinal hormones (peptides) are involved in regulating glucose metabolism through humoral gut–brain axis. Some of them are: ghrelin, neuropeptide Y (NPY), cholecystokinin - CCK, gastric inhibitory polypeptide - GIP, glucagon-like peptide (GLP) etc. (Kojima & Kangawa, 2005; King, 2011; Korner & Leibel, 2003; Neary et al, 2004 ; Young, 2005).

7. Neuro-endocrine defence to hypoglycemia

Generally, hypoglycemia is defined as a serum glucose level below (3.8 mmol/L or, 70 mg/dL). Hypoglycemia is a rare disorder, considered as pathophysiological state rather than a disease. Just as pain and fever require identification of the underlying condition, hypoglycemia warrants diagnosis of the primary disorder causing the low plasma glucose concentration.

The symptoms of hypoglycemia are not specific. For this reason it is necessary to demonstrate a low plasma glucose concentration concomitant with symptoms and subsequent relief of symptoms by correction of the hypoglycemia, i.e., Whipple's tirade. This triade can be considered to be the basis for a patient's symptoms, regardless of the cause of hypoglycemia (Service,1992). Whipple's triad considers: 1) symptoms consistent with hypoglycemia, 2) a low plasma glucose concentration, and 3) relief of symptoms after the plasma glucose level is raised. Hypoglycemia most often affects those at the extremes of age, such as infants and the elderly, but may happen at any age. Given the survival value of maintenance of the plasma glucose concentration, it is not surprising that very effective physiological mechanisms prevent or rapidly correct hypoglycemia have evolved.

Hypoglycemic symptoms are related to the brain and the sympathetic nervous system. The central nervous system requires glucose as the preferred energy substrate. Though the brain accounts for only about 10% of body weight, it uses more than 30% of blood glucose. Hypoglycemic symptoms are mediated through both central and peripheral nervous systems. Once plasma glucose concentration fall belows 3.8 mmol/L or 70 mg/dL, a sequence of events begins to maintain glucose delivery to the brain and prevent hypoglycemia.

The first of all events is the stimulation of the autonomic nervous system and, after that, release of neuroendocrine hormones (counter-regulatory or anti-insulin hormones). Peripheral autonomic symptoms (adrenergic), including sweating, irritability, tremulousness, anxiety, tachycardia, and hunger, serve as an early warning system and

preceed the central neuroglycopenic symptoms due to cerebral glucose deprivation (e.g., confusion, paralysis, seizures, and coma) (Zammitt & Frier, 2005).
A hierarchical hormonal response exists in response to decreasing blood glucose levels. As blood glucose drops, pancreatic β-cell reduce insulin secretion. If blood glucose drops further, the pancreatic α-cell secrete glucagon and the adrenal medulla release adrenaline. Both, glucagon and adrenaline, act rapidly to increase glucose availability and therefore are the two major counter-regulatory hormones. Cortisol and growth hormone are also released, but they are unable to prevent prolonged hypoglycemia withouth the preliminary actions of glucagone and adrenaline. In sensing hypoglycemia, the nutritionally deprived brain also stimulates the sympathetic nervous system, leading to neurogenic symptoms.Decreased levels of glucose lead to deficient cerebral glucose availability i.e., neuroglycopenia, that can manifest as confusion, headache, difficulty with concentration. If the symptoms are overlooked, there can be irreversible brain damage. Eventually, the patient may go into coma and death. The adrenergic symptoms often precede the neuroglycopenic symptoms and, thus, provide an early warning system for the patient, (Cryer,1997; Heijboer et., 2006).

8. Hypoglycemia as a result of inherited or acquired disorder of carbohydrates metabolism

8.1 Hypoglicemia and inborn errors of carbohydrate metabolism

Hypoglycemia is not a disease by itself, but its presence is an indication of a problematic health condition. As a relatively frequent common event in the pediatric newborn period (childhood), hypoglicemia may be a consequence of some inborn errors of carbohydrate metabolism (Service,1992; Sinclair,1979; Caraway & Watts, 1986).

8.1.1 Defects in digestion & absorption of carbohydrates

8.1.1.1 Inherited lactase deficiency (alactasia)

Enzyme lactase is necessary to digest lactose to glucose and galactose in the small intestine. Lactase deficiency is a rare congenital disorder in which infants are born without lactase. If lactase is deficient,undigested lactose enters the large intestine, where it is fermented by colonic bacteria, producing lactic acid and gases (hydrogen, methane, carbon dioxide).The gas produced creates the uncomfortable feeling of gut distention and the annoying problem of flatulence. The lactic acid produced by the microorganisms is osmotically active and draws water into the intestine, as does any undigested lactose, resulting in diarrhea. As children are weaned and milk becomes less prominent in their diets, lactase activity normally declines to about 5 to 10% of the level at birth (Gary,1978; Sinclair,1979; Tietz et al,1986). The simplest treatment is to avoid the consumption of products containing much lactose. Alternatively, the enzyme lactase can be ingested with milk products.

8.1.1.2 Lactose intolerance

Lactose intolerance is an inability to digest significant amounts of lactose due to an absence of the enzyme lactase in adult intestines. The symptoms of this disorder, which include diarrhea and general discomfort, can be relieved by eliminating milk from the diet (Maxton et al, 1990; Tietz et al,1986).

8.1.1.3 Sucrase deficiency

Enzyme sucrase decomposes disaccharide sacharose to glucose and fructose molecules. There is a number of reports of an inherited deficiency of the disaccharidases, sucrase and isomalatase, occurring within the mucosa of the small intestine. Symptoms occur in early childhood following ingestion of sucrose. The symptoms are the same as those described in lactase deficiency except that they are evoked by the ingestion of table sugar (Gary,1978 ; Sinclair,1979; Tietz et al,1986)

8.1.1.4 Glucose galactose malabsorption

Glucose Galactose Malabsorption (GGM) is a genetic disorder caused by a defect in glucose and galactose transport across the intestinal brush border membrane. Normally, lactose in milk is broken down into glucose and galactose by lactase, an ectoenzyme on the brush border, and the hexoses, having nearly identical chemical structure, are transported into the cell by the Na^+-glucose cotransporter SGLT1 (Fig 6). The mutations causing the defect in sugar transport have been identified (Gary, 1978; Wright, 1998; Wright et al, 2002; Wright et al, 2004; Wright, 2003).

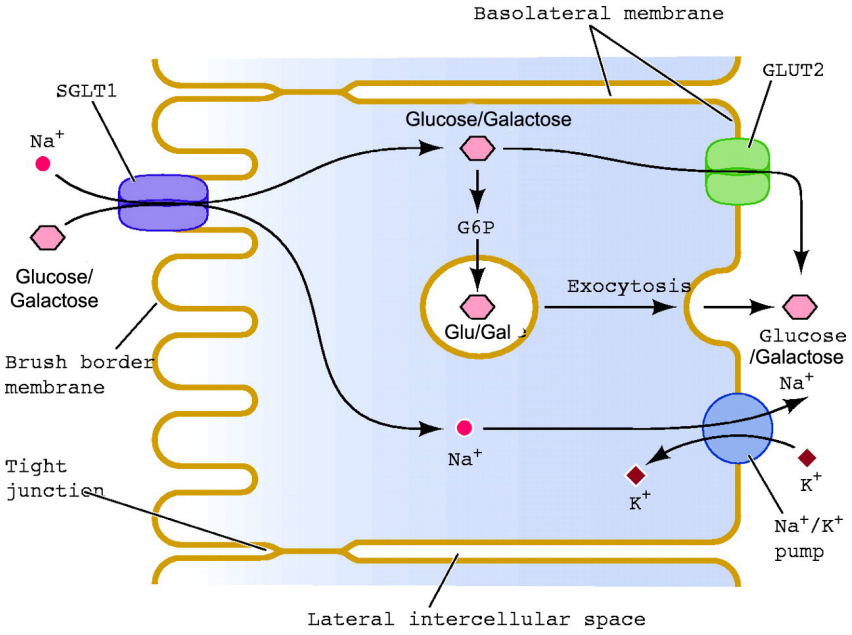

Fig. 6. Sugar transport across intestinal apithelium (Wright et al., 2004, modified)

Glucose-galactose malabsorption is an autosomal recessive disorder in which affected individuals inherit two defective copies of the SGLT1 gene, located on chromosome 22. This disease presents in newborn infants as a life-threatening diarrhea. The diarrhea ceases within 1 h of removing oral intake of lactose, glucose and galactose, but promptly returns with the introduction of one or more of the offending sugars into the diet. We conclude that mutations in the SGLT1 gene are the cause of glucose-galactose malabsorption, and sugar transport is impaired mainly because the mutant proteins are either truncated or are not

targeted properly to the cell membrane. The glucose and galactose, if left untransported, draw water out of the body into the intestinal lumen, resulting in diarrhea.
Although no cure exists for GGM, patients can control their symptoms (diarrhea) by removing lactose, sucrose and glucose from their diets. Infants showing a prenatal diagnosis of GGM will thrive on a fructose-based replacement formula and will later continue their "normal" physical development on a fructose-based solid diet. Older children and adults with severe GGM can also manage their symptoms on a fructose-based diet and may show improved glucose tolerance and even clinical remission as they age (Wright et al., 2001; Wright,1998; Gary, 1978).

8.1.2 Galactosemia

Galactose is found in the disaccharide lactose, the principal milk sugar, made from galactose and glucose. It will be recalled that galactose is required in the body, not only in the formation of milk lactose during lactogenesis in lactating mammary glands, but also as a constituent of the glucosphyngolipids (cerebrosides, globosides, gangliosides) and for the synthesis of mucopolysaccharides (MPS) or glucosaminoglycanes (GAG). Glucosaminoglycanes are linked to core proteins forming proteoglycanes. Proteoglycanes and glucosphyngolipids perform numerous vital functions within the body, some of which still remain to be studied (King, 2011; Segal, 1989).
The disruption of galactose metabolism is referred to as galactosemia. Galactosemia can result from deficiencies of three different enzymes: galactose-1-phosphate uridyl transferase (GALT), galactokinase (GALK) and uridine diphosphate galactose 4-epimerase (GALE).

8.1.2.1 Galactokinase deficiency

Galactokinase is the first enzyme in the pathway of galactose metabolism, converting galactose to galactose-1-P. The only consequence of galactokinase deficiency is the development of cataract.

8.1.2.2 Deficiency of galactose-1-phosphate uridyl transferase (GALT)

The most common and severe form of galactosemia, called classic galactosemia or Galactosemia Type 1, is an inherited deficiency of GALT, the enzyme that converts galactose-1-phosphate (galactose-1-P) to uridine diphosphate galactose (UDPgalactose). Absence or deficiency of GALT prevents the conversion of galactose into glucose in liver. People with absent or deficient GALT have intolerance to galactose.
When an infant or neonate is given milk the blood galactose level is markedly elevated (galactosemia), and galactose is found in urine (galactosuria). These can cause severe damage to eyes, kidneys, liver and brain. Afflicted infants fail to thrive. They vomit or have diarrhea after consuming milk and enlargement of liver and jaundice are common, sometimes progressing to cirrhosis. Cataracts will form, and lethargy and retarded mental development are also common. A cataract, the clouding of the normally clear lens of the eye, is a consequence of the accumulation of galactose in the lens of the eye; in the presence of high galactose amount and of aldose reductase, galactose is reduced to galactitol. The absence of the transferase in red blood cells is a definitive diagnostic criterion (Mayatepek et al, 2010).
These problems can be prevented by removing galactose and lactose from the diet. In classic galactosemia conversion of UDP-galactose to UDP-glucose is blocked. The epimerase reaction is, however, present in adequate amounts, so that the galactosemic individual can

still form UDP-galactose from glucose. This explains the normal growth and development of affected children in spite of the galactose-free diets which are used to control the symptoms of the disease (Harper, 1975).
In adults, the toxicity of dietary galactose appears to be less severe, due, in part, to the metabolism by alternative metabolic pathway of galactose-1-P by UDP-glucose pyrophosphorylase, which apparently can accept galactose-1-P in place of glucose-1-P. The levels of this enzyme may increase in the liver of galactosemic individuals, in order to accommodate the metabolism of galactose, later, after 10 years.

8.1.2.3 Uridine diphosphate galactose 4-epimerase deficiency

Uridine diphosphate galactose-4-epimerase (UDP-galactose-4-epimerase, GALE) converts UDP-galactose to UDP-glucose. Reaction is freely reversible (Fig 7). In this manner, glucose, as a unique monosaccharide in systemic blood circulation in healthy subects , can be converted to galactose in many different tissues of human body if UDP-galactose-4-epimerase is present.
Galactosemia due to epimerase deficiency is the rarest and most poorly understood form. In most patients with GALE (or epimerase) deficiency, the defect presenting with clinical features is similar to classic galactosemia. The treatment for children with generalised GALE deficiency, as with GALT deficiency is the restriction of dietary galactose.
Since galactose is an essential component of galactoproteins and galactolipids, theoretically, in generalised GALE deficiency, no endogenous production of galactose is possible, with a resulting deficiency in galactolipids and galacto-proteoglycanes production.
The most common treatment is to remove galactose (and lactose) from the diet. The enigma of galactosemia is that, although elimination of galactose from the diet prevents liver disease and cataract development, the majority of patients still suffer from central nervous system malfunction, most commonly a delayed acquisition of language skills. Females will also display ovarian failure (Sarkar et al, 2010; Segal, 1989; Walter et al, 1999).

8.1.3 The inherited abnormalities in fructose metabolism

Fructose is monosacharide found in honey and in numerous vegetables and fruits. Disacharide sucrose consists of one molecule fructose attached to a molecule of glucose. It should be mentioned that fructose constitutes the main sugar of seminal fluid. Three inherited abnormalities in fructose metabolism have been identified: essential fructosuria, hereditary fructose intolerance and hereditary fructose-1,6-bisphosphatase deficiency (Baerlocher et al,1978; Gitzelmann et al., 1989; Froesch, 1978).
After absorption by the proces of facilitated diffusion, fructose enters hepatocytes , by the portal blood. A specific kinase, fructokinase, in liver and kidney catalyzes the phosphorylation of fructose to fructose 1-phosphate. Fructose 1-phosphate is cleaved to D-glyceraldehyde and dihydroxyacetone phosphate by aldolase B, an enzyme found in the liver. D-glyceraldehyde enters glycolysis via phosphorylation to glyceraldehyde 3-phosphate catalyzed by triokinase. The two triose phosphates, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, may either be degraded by glycolysis or may be substrates for aldolase A , the enzyme which forms fructose1,6-diphosphate and hence gluconeogenesis, which is the fate of much of the fructose metabolized in the liver.

$$\text{Fructose} + \text{ATP} \xrightarrow{\textit{fructokinase}} \textit{F}\text{ructose} - 1 - \text{P} + \text{ADP}$$

$$\text{Fructose} - 1 - \text{P} \xrightarrow{\textit{aldolaseB}} \text{dihydroxyacetone} - \text{P} + \text{glyceraldehyde}$$

8.1.3.1 Essential fructosuria

Essential fructosuria is a benign metabolic disorder caused by the lack of fructokinase, which is normally present in liver and kidney cortex. The disorder is asymptomatic and it may go undiagnosed.

8.1.3.2 Hereditary fructose intolerance (aldolase B deficiency)

Hereditary fructose intolerance is a potentially lethal disorder resulting from a lack of aldolase B which decomposes fructose-1-phosphate to dihydroxyaceton-phosphate plus glycerine aldehyde. The disorder is characterized by severe hypoglycemia and vomiting following fructose or sacharose intake. Prolonged intake of fructose by infants with this defect leads to vomiting, poor feeding, jaundice, hepatomegaly, hemorrhage and eventually death. The hypoglycemia that results, following fructose uptake, is caused by fructose-1-phosphate inhibition of glycogenolysis, by interfering with the phosphorylase reaction and inhibition of gluconeogenesis at the deficient aldolase step. Patients remain symptom free on a diet devoid of fructose and sucrose(Baerlocher et al, 1978; Gitzelman at al, 1989; Odièvre et al, 1978).

8.1.3.3 Hereditary fructose-1,6-bisphosphatase deficiency

This disordered is characterized by hypoglycemia, ketosis and lactic acidosis and oftern lethal course in newborn infants. Due to enzyme defect gluconeogenesis is severely impaired. Gluconeogenis precursors, such as amino acids, lactate and ketones, accumulate as soon as liver glycogen stores are depleted (Asberg et al, 2010; Baerlocher et al, 2010; Froesch, 1978; Mortensen, 2006; Song, 2010; Zaidi, 2009).

8.1.4 The glycogen storage diseases (GSDs)

Glycogen is the storage form of glucose and is present in virtually all living cells, although the liver is primary organ for storage and subsequent release of glucose into the circulation. Glycogen biosynthesis from glucose (glycogenesis), along with the release of glucose from glycogen by the process of glycogenolysis, is highly regulated process that aids in the maintaing of normal blood glucose concentration during fasting.

During the last century, patients who have deficient activity in virtually every enzyme important in the normal synthesis, degradation or regulation of glycogen have been identified. Most of them are inherited in an autosomal recessive manner. Several inborn errors of glycogen metabolism have been described, and they result from mutations in genes that code for proteins involved in various steps of glycogen synthesis, degradation, or regulation. Glycogen storage diseases are characterized by an abnormal tissue concentration (>70 mg per gram of liver or 15 mg per gram of muscle tissue of normal or abnormal structure of glycogen**).** Hypoglycemia is the main biochemical consequence of GSD type I and some of the other GSDs. The basis of dietary therapy is nutritional manipulation to prevent hypoglycemia and improve metabolic dysfunction (Heller et al, 2008; Mayatepek et al, 2010).

All glycogenosis may divide in two groups: hepatic and muscular forms of glycogenosis. The various hepatic enzyme deficiencies are expressed primarily as hypoglycemia and hepatomegaly. In glycogenosis type I, III and VI there is limitation in the output of glucose from hepatic tissue, and hypoglycemia is an prominent laboratory sing. (Goldberg & Slonim, 1993; Heller et al, 2008; Howell, 1978; Wolfsdorf.& Weinstein, 2003).

8.1.4.1 Glycogen storage disease type I

Glycogen storage disease type I (glucose-6-phosphatase deficiency; von Gierke disease; Hepato-renal glycogenoses). GSD type I (or von Gierke disease) is an autosomal recessive disorder that is caused by deficient G6Pase activity. Glucose-6-phosphatase (G6Pase), an enzyme found mainly in the liver and kidneys, plays a critical role in blood glucose homoeostasis, providing glucose during starvation. One of the important functions of the liver and, to a lesser extent, of the kidney cortex is to provide glucose during conditions of starvation. Glucose is formed from gluconeogenic precursors in both tissues, and in the liver also from glycogen. Both glycogenolysis and gluconeogenesis result in the formation of glucose 6-phosphate, which has to be hydrolysed by G6Pase before being liberated as glucose into the circulation.

Glucose-6-phosphatase is the enzyme that catalyzes the last step of glycogenolysis and gluconeogenesis in liver and kidneys, i.e. the hydrolysis of glucose 6-phosphate to free glucose and inorganic phosphate. Its genetic deficiency is characterized by the association of hepatomegaly and nephromegaly due to the accumulation of large amounts of glycogen in these organs, with hypoglycaemia and lactic acidosis.

GSD type Ia is the most frequent form of glycogenosis, accounting for about 80% of the cases. It is caused by a lack of G6Pase activity, which is easily demonstrated by measuring the activity of this enzyme in a liver biopsy specimen. The deficiency of G6Pase activity is caused by mutations in the gene encoding this enzyme.

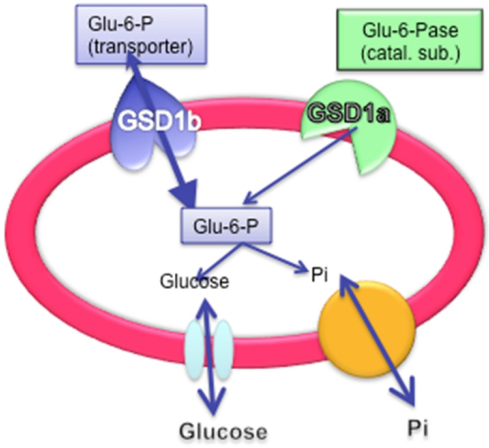

Fig. 7. The glucose-6-phosphatase system

On the basis of the imunological studies, by using the antibodies against to several of five components of glucose-6-phosphatase, GSD type I has been subcategorised into types a, b, and c, with type a as the most common, but all types have similar clinical manifestation as hypoglycemia and hepatomegaly due to the deposition of glycogen with normal structure. Fasting-induced hypoglycemia may be extreme, and with combination with lactic acidosis. Although severely affected patients may suffer brain damage in early infancy, this is not usually the case, and the most patients have normal intelligence. During starvation, however, the brain can derive energy from ketone bodies which are converted to acetyl-

CoA. It is likely that the nervous system is able to metabolise such substance as ketones and lactic acid.

Glucose-6-phosphatase consists of a hydrolase, whose catalytic site faces the lumen of the organelle and of translocases required for the transport of glucose 6-phosphate, Pi and glucose (Figure 7). Glucose 6-phosphate is transported into the lumen of the endoplasmic reticulum by a specific transporter before being hydrolysed by glucose-6-phosphatase, a transmembrane protein with its catalytic site oriented towards the lumen of the endoplasmic reticulum. Glycogen storage disease type Ia (GSD Ia) is due to a defect in glucose-6-phosphatase catalytic site and glycogen storage disease type Ib, to a defect in the glucose 6-phosphate transporter (Schaftingen and Gerin, 2002).

8.1.4.2 Debranching enzyme deficiency (type III glycogen storage disease; limit dextrinosis; Cori's disease)

Type III glycogen storage disease (amylo-1, 6-glucosidase (debrancher) deficiency) most often affects only liver, but may affect muscles as well. In this form of glycogen storage disease, a glycogen accumulates which has a structure resembling the limit dextrin produced by degradation of of glycogen by phosphorylase a , which is free of debrancher (amylo-1, 6-glucosidase) activity. Early in life, hepatomegaly and growth retardation may be striking. In contrast to patients with Type I glycogenosis, moderate enlargement of the spleen is sometime seen. Glycogen of abnormal structure frequently accumulates in muscle and heart, as well as in the liver. In the older patients it may cause a chronic progressive myopathy and cardiomegaly. With muscle involvement, the serum creatine phosphokinase (CPK) activity is elevated, and patients are usually classified as having type III b diseases. There is no renal enlargement in this disease. Generally, the clinical course of this disease is milder than that of Type I glycogenoses.

8.1.4.3 Type VI glycogenosis (hepatic phosphorylase deficiency; Hers' disease)

Large group of patients with hepatic forms of the glycogen storage diseases have increased hepatic glycogen (with normal structure) and a reduction to about 25 percent normal of hepatic phosphorylase activity. Patients with increased liver glycogen and profound reduction in liver phosphorylase activity (and normal activating system) continued to be observed. Hypoglycemia is present (Wolfsdorf & Weinstein, 2003; Heller et al, 2008).

8.1.4.4 Glycogen synthase deficiency (type 0 glycogen storage disease; GSD0)

Type 0 glycogen storage disease (GSD0) is caused by deficiency of the hepatic isoform of glycogen synthase (Weinstein et al, 2006). Although GSD0 has been classified as a glycogen storage disease, this is a misnomer. In contrast to all other types of glycogenoses, which are characterized by increased glycogen storage, deficiency of glycogen synthase causes a marked decrease in liver glycogen content. GSD0 is the only GSD not associated with hepatomegaly and hypoglycemia typically is milder than in the other types of GSD (Wolfsdorf & Weinstein, 2003; Weinstein, 2006).

Patients with GSD0 have fasting ketotic hypoglycemia. Most children are cognitively and developmentally normal. Until recently, the definitive diagnosis of GSD0 depended on the demonstration of decreased hepatic glycogen on a liver biopsy. The need for an invasive procedure may be one reason that this condition has been infrequently diagnosed. Mutation analysis of the GYS2 gene (12p12.2) is a non-invasive method for making this diagnosis in patients suspected to have this disorder.

8.1.5 Disorders of gluconeogenesis

A key role of gluconeogenesis is in the maintenance of blood sugar. Deficiency of any enzyme participating in gluconeogenesis can lead to hypoglycemia with lactic acidosis. Inborn deficiencies are known of each of the four enzymes of the glycolytic-gluconeogenic pathway that ensure a unidirectional flux from pyruvate to glucose: pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase, and glucose-6-phosphatase (van den Berghe, 1996).

8.1.5.1 Pyruvate carboxylase deficiency (PCD)

Pyruvate carboxylase, a member of the biotin-dependent enzyme family, catalyses the ATP-dependent carboxylation of pyruvate to oxaloacetate. Pyruvate carboxylase is an important enzyme in gluconeogenesis. Deficiency of pyruvate carboxylase can lead to hypoglycemia with lactic acidosis. Pyruvate carboxylase deficiency is a rare disorder that can cause developmental delay and failure to thrive starting in the neonatal or early infantile period. PCD results in malfunction of the citric acid cycle and gluconeogenesis, thereby depriving the body of energy. Based on the severity of the clinical presentation and the biochemical disturbances, two clinical forms have been described. The milder form A presents in infancy with delayed neurological development, chronic lactic acidemia and a normal lactate to pyruvate ratio. Longer survival, but with severe clinical sequelae, is common in the mild form A. The complex form B presents neonatally or in early infancy with severe metabolic acidosis, lactic acidosis, ketosis, and hepatomegaly (Jitrapakdee &. Wallace, 1999; Ahmad et al., 1999).

8.1.5.2 Phosphoenolpyruvate carboxykinase deficiency

Phosphoenolpyruvate carboxykinase is an important enzyme in gluconeogenesis. It is found in both cytosol and mitochondria of the liver cells. Deficiency of the enzyme, a rare inherited disorder, can cause severe, persistent neonatal hypoglycaemia and liver impairment (Hommes et al, 1976; Vidnes & Sovik, 1976).

8.1.5.3 Fructose-1,6-diphosphatase (FDPase) deficiency

Fructose-1,6-diphosphatase (F1,6DPase) catalyzes the conversion of fructose-1,6-diphosphate (F1,6DP) to fructose-6-phosphate. The F1,6BPase reaction is a major point of control of gluconeogenesis. Fructose-1,6-diphosphatase (FDPase) deficiency is an autosomal recessive disorder caused by a mutation of the FDP1 gene and results in impaired gluconeogenesis (Froesch, 1978; Gitzelmann et al, 1989; Hellerud, 2010).

Patients with FDPase deficiency typically present in the newborn period with symptoms or signs related to hypoglycemia and metabolic acidosis following ingestion of fructose. Patients lacking FDPase accumulate intrahepatocellular fructose-1,6-bisphosphate (FDP) which inhibits gluconeogenesis and, if intracellular phosphate stores are depleted, inhibits glycogenolysis. The inability to convert lactic acid or glycerol into glucose leads to hypoglycemia and lactic acidosis (Asberg et al, 2010; Hellerud, 2010; Song, 2011; Zaidi, 2009; Mortensen, 2006). The accumulation of fructose 1,6-diphosphate (FDP) inhibits phosphorylase a in liver, the first enzyme of glycogenoslysis.

8.1.5.4 Glycero kinase deficiency

Glycerol kinase (GK) catalyzes the phosphorylation of glycerol to glycerol 3-phosphate (G3P) which is important in the formation of triacylglycerol (TAG) and fat storage. GK is at the interface of fat and carbohydrate metabolism. GK deficiency (GKD) is an X-linked

inborn error of metabolism that is characterized biochemically by hyperglycerolemia and glyceroluria and is due to mutations within or deletions of the GK gene on Xp21 (Rahib et al, 2007). Isolated GKD can occur in patients with or without symptoms, mainly due to disturbed energy homeostasis associated with hyperketotic hypoglycemia (Sjarif et al, 2004). The greater importance of glycerol as a gluconeogenetic substrate in children than in adults may explain the episodes in young patients with GKD, often elicited by catabolic stress (Hellerud et al, 2004).

8.1.6 Leucine-sensitive hypoglycemia

This type of hypoglycemia was reported in 1956 by Cochrane who described children who became hypoglycemic on a casein-rich diet and whose symptoms worsened on feeding on high protein and low carbohydrate diet. The one-third of all infants with unexplained hypoglycemia may be sensitive to leucin. Presentation is most often in the first year of life.A common symptom is the development of convulsion after milk-feeding, (MacMullen et al., 2001) .

Leucine produces hypoglycemia by causing the release of insulin from the pancreas islet cells. The hypoglycemia found in maple syrup urine diseases is probably caused by high circulating levels of leucin. Two-thirds of the infants who have had leucine-sensitive hypoglycemia have subsequently mental retardation and neurological disorders (Roe &, Kogut, 1982; Sinclair, 1979).

8.1.7 Hyperinsulinism/hyperammonemia (HI/HA) syndrome

Hyperinsulinism is the most common cause of hypoglycemia in early infancy. Congenital hyperinsulinism, is usually caused by genetic defects in beta-cell regulation, including a syndrome of hyperinsulinism plus hyperammonemia (Kelly et al., 2001; Kogut, 1982; Stanley, 1997).

The hyperinsulinism/hyperammonemia (HI/HA) syndrome is a form of congenital hyperinsulinism in which affected children have recurrent symptomatic hypoglycemia together with asymptomatic, persistent elevations of plasma ammonium levels. The disorder is caused by dominant mutations of the mitochondrial enzyme, glutamate dehydrogenase (GDH), that impair sensitivity to the allosteric inhibitor, GTP. These data confirm the importance of allosteric regulation of GDH, as a control site for amino acid-stimulated insulin secretion and indicate that the GTP-binding site is essential for the regulation of GDH activity by both GTP and ATP (MacMullen et al, 2001; Kogut,1982).

9. Hypoglycemia as results of acquired disorders of carbohydrates metabolism

9.1 Liver and kidney disorders

Although hypoglycemia is usually linked with diabetes, there are various types of conditions, which are generally rare, that can cause it even in those who do not have diabetes. Any disorder or abnormality in the functioning of the liver can disturb the process of blood-sugar regulation, resulting in hypoglycemia. On the other hand, disorders in kidney can cause problems in excretion of certain medications. Hence, kidney disorders can be one of the major causes of low blood sugar.

9.1.1 Hypoglycemia in liver disorders

Symptomatic hypoglycemia is uncommon in liver diseases because glucose homeostasis can be maintained with as little as 20 per cent of healthy parenchymal cells, but biochemical hypoglycemia has been reported in a wide variety of aquired hepatic diseases. The hypoglycemia of Reye's syndrome and sepsis, as well as alcohol hypoglycemia, are considered to be the consequence of hepatic disturbance. Acute viral hepatitis results in serious impairment in hepatic glycogen synthesis and gluconeogenesis and frequently gives rise to fasting hypoglycemia (Felig et al., 1970). Glycogen stores rapidly disappear as liver disease (including cirrhosis due to alcoholism) progresses, causing recurrent hypoglycemia (Service, 1992).

Reye syndrome is a fatal disease, most commonly occuring following some virus infections (influenza A, influenza B, herpes, varicella zoster and several other common viral infections). Epidemiologic evidence suggests that aspirin plays a potentiating role in the pathogenesis of this syndrome. The hepatic dysfunction appears to be the primary error and the direct result of a mitochondrial disturbance that causes secondary metabolic derangement, (hyperamoniemia, hypoprotrombinemia without hyperbilirubinemia), including hypoglycemia.

Hypoglycaemia in patients with hepatocellular carcinoma usually occurs during the terminal stage of the illness, but there are patients with hepatocellular carcinoma who develop hypoglycaemia early in the course of their illness. Hypoglycemia occurs predominantly as a paraneoplastic manifestation of hepatocellular carcinoma, (Sorlini et al., 2010; Thipaporn et al., 2005; Young, 2007).

Ethanol is a potent hypoglycemic agent, causing decreased endogenous glucose production and glycogenolysis. The volume of alcohol intake is correlated with the degree of resulting hypoglycemia (Raghavan et al., 2007; Smeeks, 2008). Ethanol-induced hypoglycemia arises from inhibition of gluconeogenesis as a result of the increase in the NADH-NAD ratio, which suppresses the conversion of lactate to pyruvate, glycerophosphate to dihydroxyacetone phosphate, and glutamate to α-ketoglutarate and several tricarboxylic cycle reactions. Ethanol reduces rates of hepatic glucose production, supresses plasma insulin concentration, increases plasma lactate concentration, beta hydroxybutirate, glycerol and free fatty acids, and increases lactate-pyruvate and beta-hydroxybutirate-acetoacetate ratios. Hypoglycemia usually develops within 6 to 36 hours of the ingestion, of even moderate amounts of ethanol by persons chronically malnouriched or by healthy persons who have missed one of the meals. Healthy children are especially susceptible to ethanol hypoglycemia. Blood ethanol levels may not be elevated when the patient is hypoglycemic. Healthy children are especially susceptible to ethanol hypoglycemia. Blood ethanol levels may not be elevated when the patient is hypoglycemic(Arky, 1989; Badawy, 1977; Service, 1992).

9.1.2 Hypoglycemia in kidney disorders

Kidneys play a significant role in carbohydrate metabolism under both physiological and pathological conditions due to renal gluconeogenesis. Hypoglycemia in patients with renal failure may be due to inadequate gluconeogenic substrate availability. It seems that disturbances in renal gluconeogenesis together with lower degradation of insulin played the key role in creating hypoglycaemia in patients with renal diseases. Hypoglycemia should be suspected in any patient with renal failure who exhibits any change in mental or neurologic status (Arem, 1989; Rutsky,1978). Also, kidney disease is a frequent cause of adverse medication reactions due to the problems in excretion of certain medications and, therefore, causing hypoglycemia in older adults (Gerich, 2001).

9.2 Hormonal disturbances and hypoglycemia

Adrenocortical insufficiency or **Adrenocortical hypofunction** is defined as the deficient production of glucocorticoids or mineralocorticoids, or both. Hypoglycemia is common in adrenocortical insufficiency. Primary adrenocortical insufficiency (Addison's disease) is due to destruction of the adrenal cortex, whereas in secondary adrenocortical insufficiency impaired cortisol production is due to deficient ACTH production. Spontaneous hypoglycemia has been reported to be a frequent finding in isolated ACTH deficiency. The cause for primary adrenocortical insufficiency is autoimmune destruction or tuberculosis of the adrenal cortex.

Hypoglycemia in hypopituitarism is common in children under 6 years of age but less so beyond that age. Asymptomatic hypoglycemia has been observed in isolated growth hormone deficiency after prolonged fasting (Tyrrell, 1992).

Insulinoma. Insulin-producing tumors of pancreas can cause severe hypoglycemia; among these are islet cell adenoma and carcinoma (insulinoma). Insulinoma is uncommon in persons less than 20 years of age and is rare in those less than 5 years of age. Of the patients with insulinoma, approxymately 87 per cent have single benign tumors. These tumors are most common in women (60%), with median age of diagnosis 50 years (Service, 1992).

9.3 Reactive hypoglycemia

The post-prandial hypoglycemia occurs immediately following meals, with no known causes (idiopathic reactive hypoglycemia, RH) (Krinsley & Grover, 2007).

Alimentary hypoglycemia, another form of RH related to prior upper GI surgery (Guettier, 2006), results from rapid glucose absorption into the intestine and increased insulin secretion after every meal. The food stimulated hypoglycemia usually cause symptomes mediated by the autonomic nervous system - sweating, shakiness, anxiety, palpitations, and weakness, and rarely those of impairment of central nervous system function. The food deprived hypoglycemia, on the other hand, usually result in impariment of central nervous system functions - reduced intellectual capacity, confusion, irritability, abnormal behavior, convulsions and coma (Service,1992).

Infections. Hypoglycemia often occurs during or following acute infections in older adults. Infection-related hypoglycemia increases the risk of death and morbidity among persons over age 70 Secretion of glucagon, epinephrine, and growth hormone during hypoglycemia diminishes significantly after age 65, reducing autonomic warning symptoms in older adults.

Sepsis as a cause of hypoglycemia should be readily apparent. The mechanism for hypoglycemia with sepsis is not well defined. Depleted glycogen stores, impaired gluconeogenesis, and increased peripheral utilization of glucose may all be contributing factors. Laboratory testing can confirm the suspicion of hepatic dysfunction (Rattarasarn, 1997).

9.3.1 Hypoglycemia due to drug medications

Insulin treatment of Diabetes mellitus. Hypoglycaemia is a serious, frequent and recurrent complication of treatment of diabetes mellitus with insulin, which may become a direct danger to the patient's life. Hypoglycaemia represents the limiting factor to obtain good glycemic control. Dysregulation of counteracting mechanisms and autonomic nervous system neuropathy contribute to a strong increase in the incidence of hypoglycaemia in type 1 diabetic patients, but also in long lasting type 2 diabetic patients (Cryer, 2001, 2008).

Predictors of hypoglycemia in patients with type 2 diabetes include treatment with insulin and duration of insulin treatment, a history of previous hypoglycemia. Primary risk factors for hypoglycemia in decreasing importance have been reported as age over 64, current insulin treatment, sulfonylurea treatment, polypharmacy, renal impairment and previous hypoglycemic episodes (Miller et al., 2001).

Antibiotics. Pentamidine used in treating opportunistic infections associated with immunosuppression (e.g. Pneumocystis pneumonia) and protozoan parasites, causes severe hypoglycemia by increasing insulin secretion. Isoniazid causes hypoglycemia through cytotoxic hepatic damage (Service, 1992).

Sulfonamides and Fluorquinolones have been known to cause significant, life-threatening hypoglycemia by increasing insulin secretion.

Cardiac medications. Beta-blockers inhibit glycogenolysis and are most likely to be associated with hypoglycemia in older adults. Isolated reports indicate that angiotensin-converting enzyme inhibitors can cause hypoglycemia by increasing insulin sensitivity.

Salicylates. It has been determined more recently that salicylates, such as aspirin, decrease serum glucose by reversing or inhibiting the process of insulin resistance related to generalized inflammatory responses.

Psychotropic medications. It should be avoided haloperidol in older adults with a history of hypoglycemia, or sulfonylurea or insulin use, due to the risk of severe hypoglycemic interactions. Tricyclic antidepressants, chlorpromazine, MAO inhibitors, and lithium also have been reported to cause severe hypoglycemia.

Quinolines. Quinines, used as an anti-malarial and anti-arrhythmic agents, have strong hypoglycemic properties, increasing insulin secretion as the sulfonylureas do (Service, 1992).

10. Conclusion

Hypoglycemia is defined as a serum glucose level below (3.8 mmol/L or, 70 mg/dL). The plasma glucose level is tightly controlled throughout life in the normal individual. The stability of the plasma glucose level is a reflection of the balance between the rates of whole body glucose production and glucose utilization. The physiological post absorptive serum glucose concentration in healthy humans range is 4.4-5.*8* mmol/L (80 to 110 mg/dL). Variation in blood glucose levels above or below the normal range usually indicate to serious diseases. Even mild disruptions of glucose homeostasis can have adverse consequences.

Hypoglycemia most often affects those at the extremes of age, such as infants and the elderly, but may happen at any age, in neonatal period and early childhood As a relatively frequent common event in the pediatric newborn period (childhood), hypoglicemia may be a consequence of some inborn errors of carbohydrate metabolism. In adults, hypoglycemia is a result of acquired disorders, primarily due to disturbance of physiological function of some organs (liver, kidney, CNS) or manifests disorders of some endocrine glands, involved in carbohydrate metabolism .

The symptoms of hypoglycemia are not specific and are related to disturbance of the brain and the sympathetic nervous system. The stimulation of the autonomic nervous system produces sweating, pale skin, irritability, anxiety, weakness, hunger, nausea, serving as an early warning system and preceding the neuroglycopenic symptoms due to cerebral glucose deprivation, e.g. headache, confusion, inability to concentrate or pay attention, mental confusion, difficulty in thinking, changes in vision, lethargy, sleepiness, stupor.

Biochemical hypoglycemia has been reported in a wide variety of acquired hepatic and renal diseases. Also, kidney disease is a frequent cause of adverse medication reactions due to the problems in excretion of certain medications and, therefore, causing hypoglycemia in older adults due to drug medications**.** Hypoglycaemia is a serious, frequent and recurrent complication of diabetes mellitus treatment with insulin, which may become a direct danger to the patient's life.

Laboratory diagnosis of hypoglycemia is very important in medical praxis, especially in pediatric praxis, suggesting some inborn errors of carbohydrate metabolism, or, in adults, suggesting hepatic disorders, renal failure, and cardiac disorders, neuropsychiatric disorders, etc.

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6

Autoimmune Associated Diseases in Pediatric Patients with Type 1 Diabetes Mellitus According to HLA-DQ Genetic Polymorphism

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1. Introduction

Diabetes mellitus type 1 is the most common endocrine metabolic disorder in childhood and adolescence. In this condition there is an absolute insulin deficiency secondary to progressive destruction of pancreatic beta cells, causing severe alterations in the metabolism of all essential elements (carbohydrates, lipids and proteins).

The most obvious alteration is chronic hyperglycemia, which is essential to diagnose the disease, and moreover, is the main responsible of many vascular and neurological complications that diabetic patients may develop long-term.

In the development of diabetes mellitus type 1 involving both genetic and environmental factors. The traditional concept is that environmental factors may act as triggers of the immune response against β of Langerhans cell phenotype in a genetically predisposed to the development of diabetes mellitus type 1.

Autoimmune diseases are syndromes caused by activation of T cells or B or both, without evidence of other causes such as infection or cancer. When dendritic cells expressing self-antigens in the context of HLA molecules stimulate peripheral T cells, they do so that they remain alive but anergic, no response until they contact a dendritic cell with multiple moleculescostimulatory expressing microbial antigens.

Although many autoinmumnes diseases characterized by abnormal production of pathogenic autoantibodies, most of it is caused by an overreaction, combined T and B cells. In animal models of type 1 diabetes mellitus have demonstrated the high expression of MAdCAM-1 and GlyCAM-1 on HEV (high endothelial venules) of the inflamed pancreatic islets and the treatment of animals with inhibitors of the L-function selectin and α4 integrin, blockeddevelopment of type 1 diabetes mellitus.

The American Diabetes Association divides the type 1 diabetes mellitus in two subgroups: 1A is the result of autoimmune destruction of beta cells and the 1B subtype, which do not immunomarkers indicating a destructive autoimmune process of beta cellspancreas. However develop insulin deficiency by unidentified mechanisms and are prone to ketosis. It has been a predominance of African Americans and Asians in the 1B subtype.

Genetic studies have shown that it is an inherited disease with polygenic trait. Genome wide studies have indicated the presence of at least 20 chromosomal regions that may contribute to genetic predisposition to type 1 diabetes mellitus. The most important genes that

influence susceptibility to type 1 diabetes are located in the HLA complex located on the short arm of chromosome 6 (6p21.3).

The HLA-DQ is the locus that confers the major genetic susceptibility to develop type 1 diabetes in humans. DQ molecules have two chains, alpha and beta, encoded by the DQA and DQB genes. Susceptibility to type 1 diabetes mellitus has been described by combining alpha-beta chains with no amino acid aspartic acid at position 57 of beta chain (DQB1*0302 linked to DR4 and DQB1*0201 linked to DR3) and the presence of the amino acid arginine at position 52 of the alpha chain (DQA1*0301 linked to DR4 and DQA1*0501 linked to DR3).

Based on these findings have been described both genotypes DQA/DQB disease associated with DQA1*0501/DQB1*0201 and DQA1*0301/DQB1*0302, which is more specific than DR3/DR4 genotype.

Although other types of islet cells and α cells (glucagon producing), delta cells (somatostatin producing) cells or PP (pancreatic polypeptide-producing) are functionally and embryologically similar to beta cells and express the most same. These proteins, inexplicably are free of autoimmune process.

From the pathological standpoint, the cells of the pancreatic islets are infiltrated by lymphocytes (a process called insulitis). After the destruction of beta cells, the inflammatory process forwards, the islets are atrophic and disappear immunomarkers.

Insulitis studies in humans and in animal models of type 1 diabetes mellitus (NOD mouse and BB rat) have identified the following abnormalities in both humoral branch as in the immune system cells:

1. Autoantibodies against cell islet.
2. Activated lymphocytesin islets, peripancreatic lymph nodes and widespread circulation.
3. T lymphocytes that proliferate when stimulated with islet proteins.
4. Release of cytokines within the insulitis.

The beta cells appear to be particularly vulnerable to the toxic effect of some cytokines (tumor necrosis factor), interferon gamma and interleukin 1. Precise mechanisms are unknown the death of beta cells, but may involve formation of nitric oxide metabolites, apoptosis and direct cytotoxic effects of CD8+ T cells. It is believed that the destruction process does not involve autoantibodies against islet cells, since these antibodies do not react in general to the surface of islet cells and are capable of transferring diabetes mellitus in animals.

Among islet molecules that are targets of the autoimmune process are insulin, glutamic acid decarboxylase (glutamic acid decarboxylase, GAD), the biosynthetic enzyme of the neurotransmitter gamma aminobutyric acid (gamma-aminobutyric acid,GABA), ICA-512/IA-2 (with homology to tyrosine phosphatases, and fogrina (protein in secretory granules of insulin). Other less precisely defined autoantigens are islet ganglioside and carboxypeptidase H. Except none of the insulin-specific autoantigens are beta cells, which makes us wonder how these are destroyed selectively.

Current theories favor the onset of an autoimmune process directed against beta cell molecule, which then spreads to other islet molecules as the autoimmune process destroys the beta cells and creates a series of secondary autoantigens. Beta cells of individuals with type 1 diabetes mellitus do not differ from the beta cells of normal people, because the transplanted islets are destroyed by the recurrence of autoimmune process of type 1 diabetes mellitus.

Autoantibodies against islet cells (ICA) is a combination of several different antibodies directed against islet molecules such as GAD, insulin, IA-2/ICA-512 and islet ganglioside and serve as a marker of the autoimmune process of type 1 diabetes mellitus. The determination of the ICA may be useful to classify as type 1 diabetes mellitus and nondiabetic individuals identify risk. The ICA is present in most (> 75%) of individuals newly diagnosed with type 1 diabetes mellitus in a significant minority of diabetics newly diagnosed type 2 (5-10%) and sometimes, in pregnant women with gestational diabetes (<5%).

In 3-4% of first-degree relatives of individuals with type 1 diabetes mellitus ICA exist. Along with the presence of a disorder of insulin secretion in the proof of intravenous glucose tolerance predict a 50% higher risk of developing type 1 diabetes mellitus in the next 5 years. Without this disorder of insulin secretion, the presence of ICA predicts a five-year risk <25%. After this it follows that the risk of a first degree relative of type 1 diabetes mellitus is low. Today no approved treatment to prevent development of type 1 diabetes mellitus, so the detection of ICA in non-diabetic population has not been established as screening.

There is another theory that talks about environmental factors as triggers of the autoimmune process in genetically vulnerable patients, but it is difficult to find an environmental trigger, since the event may precede by several years the development of the disease. Among the hypothetical environmental triggers include viruses (coxsackie and rubella), proteins early exposure to cow's milk and nitrosoureas.

A major advance would get delay or prevent diabetes, there have been some intervention in animal models, whose main objective has been the immune system (immunosuppression, selective deletion of T cell subsets, immune tolerance induction to proteinsisland), while others avoid the death of islet cells by blocking the cytotoxic cytokines or increasing islet resistance to the destruction process.

Type 1 diabetes mellitus is often associated with autoimmune diseases. Thus, there has been an increased prevalence of autoantibodies related to celiac disease and many other autoantibodies against endocrine and nonendocrine organs. Not infrequently, these diseases manifest themselves associated paucisymptomatic and are diagnosed late. Despite this apparent relationship between type 1 diabetes mellitus and these autoimmune diseases has not been shown that the degree of glycemic control influence the likelihood of developing or subsequent developments. We must not forget that many of these clinical situations produce per se a decrease in the expectancy and quality of life of patients, another reason to actively pursue and establish treatment as soon as detected.

Autoantibodies can be found in up to 25% of children and adolescents with diabetes, but only 3-5% have hypothyroidism. Hyperthyroidism is less common, but more often than children without diabetes.

Autoimmune hypothyroidism may be associated with goitre (Hashimoto's thyroiditis or goiter), or in later stages of the disease, minimal residual thyroid tissue (atrophic thyroiditis). Because the autoimmune process gradually reduces thyroid function, there is a compensatory phase during which thyroid hormone levels are maintained by an elevated TSH.

Although some patients may have mild symptoms, this phase is called subclinical or mild hypothyroidism. Later, T4 levels fall and TSH levels increase even more, the symptoms become more obvious at this stage (usually TSH> 10 mU/L) is called clinical hypothyroidism.

In Hashimoto's thyroiditis, there is a marked lymphocytic infiltration of the thyroid with germinal center formation, atrophy of thyroid follicles accompanied by oxyphil metaplasia, absence of colloid and mild or moderate fibrosis. As with most autoimmune disorders, susceptibility to this type of hypothyroidism depends on a combination of genetic and environmental factors and is increased sibling risk of autoimmune hypothyroidism or Graves disease.

Genetic risk factors for this type of hypothyroidism in subjects caucasians are HLA-DR polymorphisms, specifically the HLA-DR3, HLA-DR4 and HLA-DR5. There is also a weak relationship between polymorphism of CTLA-4, a gene regulating T cells and autoimmune hypothyroidism. HLA-DR polymorphisms and CTLA-4 constitute about half of cases hipotiroidsimo autoimmune susceptibility. It is still necessary to identify other contributing loci. A gene located on chromosome 21 could be the cause of the relationship between autoimmune hypothyroidism and Down syndrome.

The lymphocytic infiltrate thyroid autoimmune hypothyroidism is composed of CD4+ T cells and activated CD8+ and B cells. It is believed that the destruction of thyroid cells is mediated in a primary CD8+ T cells cytotoxic, which destroy their targets by perforin that cause cellular necrosis or through granzyme B, which induces apoptosis.

Addition, cytokine production by local T cells, such as tumor necrosis factor, IL-1 and interferon gamma, can return to thyroid cells more susceptible to apoptosis mediated by death receptors such as Fas, which activate their ligands respective T cellIn addition, these cytokines directly disrupt the function of thyroid cells, and induce the expression of other proinflammatory molecules by thyroid cells themselves, such as cytokines, molecules of HLA class I and II, adhesion molecules, CD40 and nitric oxide.

The administration of high concentrations for therapeutic cytokines (notably IFN alpha) is associated with enhancement of autoimmune thyroid disease, possibly by mechanisms similar to those involved in sporadic disease.

The Tg and TPO antibodies are markers of thyroid autoimmunity with clinical utility, but their pathogenic effect is limited to a secondary role in the amplification of a developing immune response. TPO antibodies fix complement and are complex to the complement membrane attack the thyroid gland in case of autoimmune hypothyroidism. However, the transplacental passage of anti-Tg antibodies or anti-TPO has no effect on fetal thyroid gland, indicating that it takes an injury mediated by T cells to initiate autoimmune injury of the gland.

Although it has been associated with the presence of subclinical hypothyroidism with an increased risk of symptomatic hypoglycemia and hipocrecimiento, it is quite common for thyroid dysfunction clinically pass unnoticed, so you must determine the levels of thyrotropic hormone (TSH) annually in those without autoantibodies or more often if there are or the patient has any symptoms.

Celiac disease is 10 times more common in diabetics than in the general population and may affect up to 1-10% of diabetic patients. Also known as celiac sprue or gluten sensitive enteropathy is an autoimmune disorder triggered by ingestion of gliadin fractions present in the gluten and similar proteins of rye and barley in genetically predisposed individuals.

Gluten is the main protein component of wheat, rye and barley. In celiac disease is triggered by an immune reaction that leads to inflammation of the small intestine mediated by T lymphocytes, with the development of hyperplastic crypts, intraepithelial lymphocytes and

villous atrophy, causing a chronic enteropathy with a broad range of manifestations, which make a systemic disease of varying severity.
Adherence to a gluten-free diet is followed by clinical and histological improvement in these patients, with normalization of long-term intestinal architecture, and the property of the recurrence of symptoms when gluten is reintroduced in the diet .
The presence of an immune component in the etiology of the disease was suspected for three reasons. First, no serum IgA antigliadin antibodies and endomysial, although it is unclear whether primary or secondary to tissue injury. Endomysial antibody has a sensitivity of 90 and specificity 95%, and its antigen is tissue transglutaminase. Secondly, treatment with prednisolone for four weeks in a celiac patient who continues to eat gluten induces remission and duodenal epithelium gives a more normal level. Finally, the gliadin peptides interact with gliadin-specific T cells, which in turn can act as mediators of tissue injury or cause the release of one or more cytokines that are responsible for tissue injury.
In celiac disease are also implicated genetic factors, its incidence varies widely among different population groups (high in Caucasians and low in color and eastern race) and is 10% in first degree relatives of patients with celiac disease. In addition, about 95% of celiac patients express the allele of the human leukocyte antigen DQ2, whereas only a minority of all people who express DQ2 have celiac disease.
Not forget that the diagnosis of celiac disease is made by biopsy of the small intestine. Is performed on patients with symptoms and laboratory findings suggestive of malabsorption or lack of nutrients.
Is often asymptomatic, but can cause gastrointestinal symptoms, short stature and anemia. This condition is also associated with an increased number of hypoglycemic episodes and a progressive decrease in insulin requirements in the year prior to diagnosis. It is also recommended measuring endomysial or tissue transglutaminase after diagnosis and every 2-3 years in post, in asymptomatic patients or whenever there is clinical suspicion, given the possibility of seroconversion over time in some patientsin which antibodies were initially detected.
Addison's disease, another autoimmune disease is present up to 2% of type 1 diabetes presenting autoantibodies against the enzyme 21-hydroxylase and the enzyme cleavage of the side chain, but it ignores the importance of these antincuerpos in the pathogenesis ofadrenal insufficiency. Some antibodies cause adrenal insufficiency by blocking the binding of ACTH to its receptors.
The appearance of two or more of these autoimmune endocrine same person characterized in a polyglandular autoimmune syndrome type II (thyroid, parathyroid and gonadal tissue), this syndrome has yet mutated gene on chromosome 6 and is associated with alleles B8 and HLA DR3.
The presence of adrenal insufficiency is rare, so it is recommended not look systematically. In addition to the classic symptoms for adrenal insufficiency are at risk of frequent hypoglycemia and reduced daily insulin requirements.
The 15-20% of adults have diabetes autoimmune gastropathy presenting autoantibodies to gastric parietal cells, and 50% have clinical or pathological signs of atrophic gastritis. Yet there are no recommendations regarding the detection of these antibodies, given the lower prevalence in childhood.
With respect to autoimmune diseases of the skin, vitiligo has been found up to 7% of children and adolescents with type 1 diabetes mellitus.

The objectives of this study are the following: make an epidemiological study of type 1 diabetes mellitus in childhood and adolescence, to study the HLA-DQ genetic group and general parameters in the onset of the disease, and the pursuit of development of autoimmune diseases.
Today diabetes education is fundamental and essential, in consultation diabetes control emphasizes good glycemic control in order to reduce microvascular complications, rare in children because their development is in adulthood. It also reports on the association with microvascular problems such as diabetic retinopathy, microalbuminuria leading to nephropathy, and diabetic neuropathy, as well as on macrovascular problems such as atherosclerosis.
Are known to coexist in these patients, with chronic hyperglycemia in cardiovascular risk otrosfactores. Diabetes education programs and health promotion should report on the harmful effects of some of them, such as smoking, overweight or sedentary. We should not forget the high prevalence of these diseases justifies the systematic implementation of its screening in the units of pediatric endocrinology. The early diagnosis of these can improve the control of type 1 diabetes mellitus.

2. Patient and methods

This study was carried out at the Department of Pediatrics, General Hospital of Ciudad Real. This work is a descriptive epidemiological study on 129 children and adolescents under age 16 with type 1 diabetes mellitus, studied in this hospital since 1990. With regard to epidemiological studies by our group in the province of Ciudad Real, with an estimated total of 423 patients with DM1, of which 204 are under 16 years, the size of the sample makes it representative of the distribution of type 1 diabetes mellitus population.
The analysis began in January 2003 starting with a retrospective study of patients and performing a 3-year prospective follow-up on these patients and patients who were going to the Department of Pediatrics at the start of his diabetes. The analysis and recruitment was completed in December 2007. This study was approved by the Research and Ethics Committee of the General Hospital of Ciudad Real. Reported and informed consent was obtained from parents or guardians.
In our study we asked whether there is a relationship between the occurrence of autoimmune diseases in pediatric patients with type 1 diabetes mellitus, with the HLA-DQ genetic group. Main objective genetic group analyzed HLA-DQ by molecular biology of our patients. According to these HLA-DQ haplotypes have organized groups I, II and III, considered by the usual bibliography and diabetogenic risk.

- **Group I:** HLA-DQA1*0501/DQB1*0201
- **Group II:** HLA-DQA1*0301/DQB1*0302
- **Group III**: HLA-DQA1*0501,*0301/HLA-DQB1*0201,*0302
- **Group IV:** No genetic group associated with DM1

As secondary objectives we analyzed the disease onset general parameters such as sex and age. We collected data on whether patients had autoimmune disease associated with type 1 diabetes mellitus, if the onset of the disease has been before or after the debut of type 1 diabetes mellitus and the median time to onset of the disease. These parameters are studied during a follow-up period of 3 years with updates every 6 months. All of these secondary objectives relate them to the diabetogenic risk group assigned to each of our patients.

The degree of innovation under our study was that the determination of HLA-DQ alleles was performed by molecular biology techniques, to avoid large differences, about 50% errors (58% according to the results of our group), which are generated if only theserological determinations. HLA-DQ alleles were determined by reaction polymerase chain, with allele specific amplification (PCR-SSP). We used specific primers for DQA1 and DQB1 genes (Protrans, Ger.) Amplified products were separated by agarose gel electrophoresis in 2% and were assigned allele specific amplification.
Determinations of antithyroid antibodies, and antithyroglobulin antimicrosomal performed by chemiluminescence, IMMULITE 2000 (Dipesa ®). Thyroid hormones: T4 and TSH by chemiluminescent immunoassay technology microparticles, ARCHITECT (Abbott ®).
As serologic marker in detecting celiac disease transglutaminase antibodies were analyzed by enzyme immunoassay with recombinant human tissue transglutaminase (Eurospital ®) with a sensitivity around 95% and a specificity above 95% for populationspediatric. Patients with positive markers underwent a biopsy of the duodenum and subsequent intestinal pathology.
For the analysis of the data is first created a database with Microsoft Access and have subsequently be exported for statistical analysis by SPSS for Windows, version 12.0. We conducted a 6x4 design. Each patient was studied in 6 different moments of its evolution, with 4 viable possibilities, different genetic risk groups.
Study possible changes in the variables under study during the monitoring period, if the changes are influenced by risk group HLA-DQ, and if it influenced other control variables such as sex, age, etc. All statistical tests were performed with a significance level of 95% and an alpha of 0.05%.

2.1 Results

According to the HLA-DQ haplotypes obtained, the distribution of diabetogenic risk groups was as follows: group I (HLA-DQA1*0501/DQB1*0201): 45 patients, accounting for 34.9%, group II (HLA-DQA1*0301/DQB1*0302): 38 patients, representing 29.5%, group III (HLA-DQA1*0501,*0301/HLA-DQB1*0201,*0302): 38 patients, representing 29.5%, and group IV (no gene associated with DM1 group): 8 patients, representing 6, 2% (Figure 1).

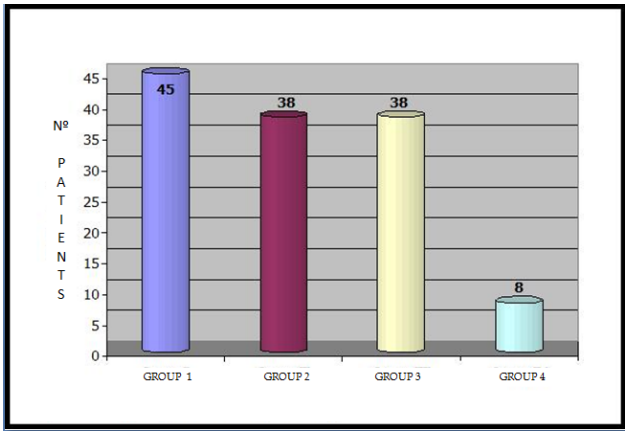

Fig. 1. HLA-DQ Diabetogenic risk groups

The gender distribution of our study population of 129 patients was as follows: males 67 patients representing 51.9% and women 62 patients representing 48.1%, with a ratio child of 1,08.

The distribution of patients by age at onset was as follows: 0 to 4 years 32 patients representing 24.8%, between 5 and 9 years 67 patients, representing 51.9%, between 10 and 14 years 29 patients who account for 22.4% and between 15 and 16 years 1 patientrepresents 0.8%. The mean age of patients, whose mean values (mean ± SD), expressed in years, diabetogenic risk groups were as follows: Group I (8.6 ± 3.4), Group II (7 ± 3), Group III (6.1 ± 2.7) and Group IV (6.8 ± 2.6). In our study found significant differences in age at debut by diabetogenic risk groups, age at onset being significantly lower in group III with group I (Figure 2).

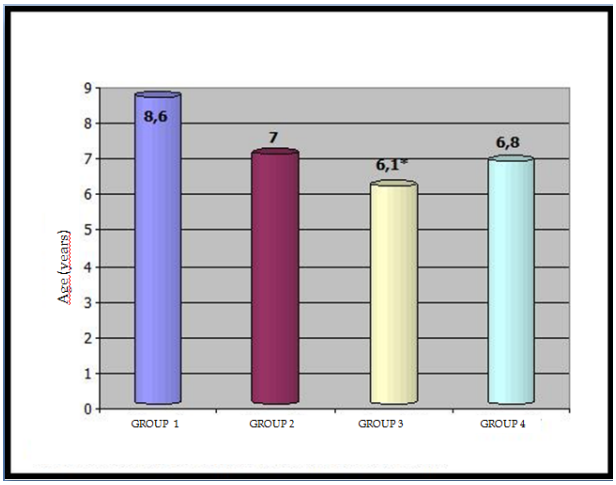

Fig. 2. Distribution of patients by age at onset

The incidence of patients with autoimmune thyroiditis in our series is 15.5%, representing a total of 20 patients. At the time of diagnosis of thyroiditis, 85% were euthyroid autoimmune thyroiditis and 15% had undergone an underactive thyroid. In the euthyroid, 17.6% (n = 3) associated with thyroid hypofunction later.

The sex distribution is as follows: 55% (n = 11) were women and 45% (n = 9) were male. No significant differences were observed in the distribution of autoimmune thyroiditis by sex.

Distribution diabetogenic risk groups was as follows: Group I: 6 patients, Group II: 5 patients, Group III: 8 patients, and Group IV: 1 patient. No significant differences were found in the diagnosis of autoimmune thyroiditis diabetogenic risk groups (Figure 3).

When analyzing patients with autoimmune thyroiditis, it was observed that 76.4% started after the debut of type 1 diabetes mellitus, whereas 23.6% were diagnosed simultaneously with the debut of it. By contrast patients with underactive thyroiditis, 28.6% presented prior to the commencement of type 1 diabetes mellitus, 42.8% thereafter, and 28.6% to debut simultaneously do the same.

In our series we found a case of hyperthyroidism in a 11-year-old was diagnosed with type 1 diabetes three years ago. The frequency of patients with celiac disease associated with type 1 diabetes mellitus in our series is 6.2%, which corresponds to 8 patients. The sex

distribution is as follows: 75% (n = 6) are women and 25% (n = 2) are male. We found significant differences in favor of women (p <0.001).

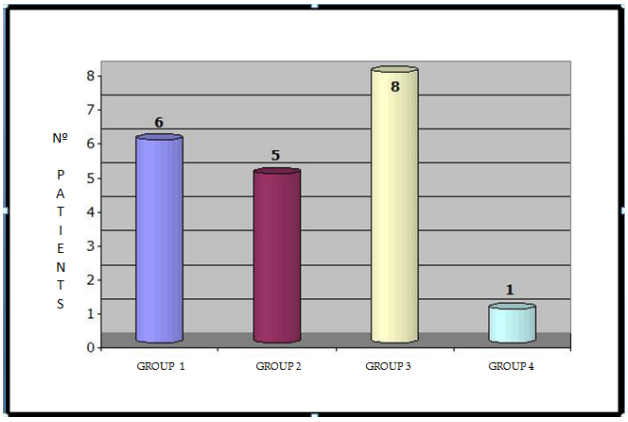

Fig. 3. Autoimmune thyroiditis by diabetogenic risk groups

Distribution diabetogenic risk groups was as follows: Group I: 2 patients, Group II: 2 patients, Group III: 3 patients, and Group IV: 1 patient. No significant differences were found in the diagnosis of celiac disease by diabetogenic risk groups (Figure 4).

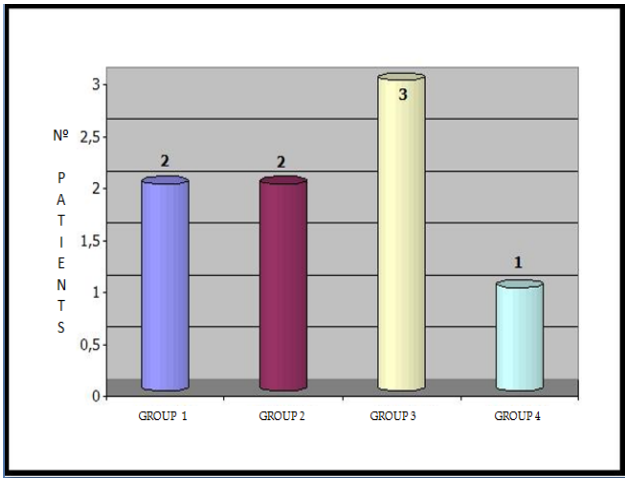

Fig. 4. Celiac disease by diabetogenic risk groups

As for the timing of the debut has been observed that in 75% (n = 6), the debut of celiac disease after debut of type 1 diabetes mellitus, and 25% (n = 2) the onset is earlier. In most cases of celiac disease were asymptomatic at diagnosis and only observed the existence of signs of malabsorption in 1 patient, abdominal distention and diarrhea. The average time between debut and diagnosis was 22 months with a minimum of 6 months and a maximum of 34 months.

In our series, 2 patients are associated with type 1 diabetes mellitus, celiac disease and autoimmune thyroiditis (1 patient 1 patient euthyroid and hypothyroid).
In 13 patients (10.1%) were type allergic processes associated allergic rhinitis and conjunctivitis, 8 patients (6.2%) had asthma, 8 patients (6.2%) were diagnosed with atopic dermatitis and 1 patient (0.7%) of vitiligo.

2.1.1 Discussion

In type 1 diabetes mellitus there is a polygenic susceptibility. The most important genes that influence human susceptibility to type 1 diabetes mellitus are located in the complex HLA class II. In our series, these data are corroborated, and that these associations are most common. 93.8% of our patients with type 1 diabetes mellitus corresponds to the genetic risk groups as has been described elsewhere, so a 6.2% suffer from type 1 diabetes mellitus without belonging to a group of HLA-DQ risk.
Our results agree with others, as published by the EURODIAB indicating that in most mediterranean countries the male/female ratio is around 1, with a slight male predominance, but with no significant differences between them.
The period of highest incidence in the study is between 5 and 9 years. These findings are consistent with studies in other countries, which show a tendency for the disease much earlier debut. In our study found significant differences in age at debut by diabetogenic risk groups, age at onset being significantly lower in group III with group I. These results indicate that the combination of different molecules in susceptibility, heterozygous in group III, accelerating the destruction of beta cells by promoting an early onset of type 1 diabetes mellitus.
Autoimmune disease most often associated to type 1 diabetes mellitus is an autoimmune thyroid disease. Our results, 15.5% compared to the percentage of subjects who agree thyroiditis associated with many studies, such as in Spain by Roland and cols (1999) or made in Italy by Lorini and cols (1996). However, some international studies show higher prevalence, such as that conducted by Lindberg et al (1997), with a prevalence of 38%.
As in other studies in our series of cases in which thyroiditis manifested clinically, it is in the form of an underactive thyroid. Although thyroid status of the majority of subjects with positive markers is euthyroid.
In our series we found a case of hyperthyroidism in a 11-year-old was diagnosed with type 1 diabetes three years ago. Hyperthyroidism is associated with type 1 diabetes mellitus present in 1% of cases, most often in adults. Other studies indicate that hyperthyroidism is usually diagnosed before or while type 1 diabetes mellitus.
Studies show that thyroiditis is more prevalent in diabetic girls than in boys. However, our results do not indicate such a difference, matching other studies.
The prevalence of celiac disease associated with children with type 1 diabetes mellitus varies between 1-16%. In our series, we found a total of 8 patients under 6.2% of all patients. These results are consistent with those of Vitoria et al. However, some studies show minor incidents, such as by Barera and colleagues (2002) with a prevalence of 3.9%. In some other series have reported higher frequencies, between 8 and 12.3%.
As for the timing of the debut our results coincide with those published by Barera et al, and Holmes et al (2002), in which the majority of patients the diagnosis of celiac disease is posterior to that of type 1 diabetes mellitus. The time elapsed since the debut of the type 1 diabetes mellitus and identification of antibodies in our series are consistent with the results

of Saukkonen et al (1996), which are located around the 2 years following the onset of type 1 diabetes mellitus. Other studies such as Maki and colleagues (1995), who observed a lower average interval around 13 months. Our results on the significant association in women are endorsed by other studies in this regard, as published in Spain by Roldan et al (1998).
Most patients were asymptomatic at the time of his presentation and noted that there were no signs of frank malnutrition. Were diagnosed by serological screening and subsequent confirmation with intestinal biopsy. Our results are consistent with those of Barrera et al.
Not found in our series more partnerships with other autoimmune diseases such as pernicious anemia, Addison's disease, Sjögren syndrome, alopecia areata, and rheumatoid arthritis.
Among the background approximately 10% of the patients had atopy and bronchial asthma. These data are consistent with those reported by Lopez Medina et al in their series. The presence of vitiligo in our series (0.7%) is lower than that observed in other studies like the one made in Italy by Romano et al (1998), showing a prevalence of 9%.

3. Conclusion

The major autoimmune diseases, autoimmune thyroiditis and celiac disease are more prevalent in our diabetic patients than in the nondiabetic population. Although we found more patients in risk group III, no significant differences with other groups.
In conclusion, these data support the recommendation that from the moment of diagnosis of type 1 diabetes mellitus regular determination of thyroid antibodies and celiac disease related. Current recommendations are vague as to what should be the most appropriate timing for this in pediatric patients. We must try to detect such diseases early, but that does not justify excessive and unnecessary repetition of diagnostic tests. The use of standardized monitoring protocols is becoming increasingly necessary to ensure better health care for children and adolescents with type 1 diabetes mellitus.

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Part 2

Cardiovascular Complications

7

Etiopathology of Type 1 Diabetes: Focus on the Vascular Endothelium

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1. Introduction

Type 1, or insulin-dependent diabetes results from the destruction of insulin-producing β-cells in the pancreas. It typically occurs in previously healthy children, being one of the most common childhood diseases in modern time. Intriguingly, the diabetes morbidity continues to rise in most parts of the world, but the causes of this development remain elusive (the Diabetes Mondial (DIAMOND) Project Group, 2006).

Many of the patients with type 1 diabetes may develop particularly in adult life severe complications encompassing both the microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (myocardial infarction, stroke and peripheral artery disease) system. The main underlying mechanism in the large vessels is represented by accelerated atherosclerosis with subsequent narrowing of the vessels and potential risk for plaque rupture. In smaller, as in the large vessels, severely impaired function of the inner layer (so-called endothelium) has been detected, and correlated with the risk of developing vascular complications, suggesting it to play a major role in precipitating the vascular disease.

It is now well established that this widespread vasculopathy develop many years before the onset of vascular complications, perhaps even before the outbreak of diabetic hyperglycemia. What exactly drives this process remains speculative, but increasing evidence suggest that factors thought to trigger type 1 diabetes may as well be harmful for the vessels. Furthermore, as delineated below, the microvascular network surrounding the islets in the pancreas appears to have important contribution to the injury process that ultimately leads to type 1 diabetes. Further multidisciplinary efforts are warranted in order to better understand the mechanism of this disease and its complications.

2. Epidemiology and pathogenesis of type 1 diabetes

There is a widespread global variation in the incidence and prevalence of type 1 diabetes. In a report by Karvonen et al. from 2000, age-adjusted incidences ranged from a low of <1/100,000 per year in China and South America to a high of >20/100,000 per year in Sardinia, Finland, Sweden, Norway, Portugal, the United Kingdom, Canada, and New Zeeland. The incidence increased with age in most of the populations with the highest incidence observed in children 10-14 years of age. An updated report from 2006 (the

DIAMOND Project Group, 2006) showed trends of increased incidence annually across the world in the populations studied (4.0% in Asia, 3.2% in Europe, and 5.3% in North America) with the exception of Central America and the West Indies were the trend was a decrease of 3.6%. The rate of increase in incidence cannot be explained by genetic shift in such a short period of time. An increasing burden of environmental factors, probably along with increased immune-mediated sensitivity of pancreatic cells to gene-environment interaction, could account at least in part for the rising prevalence of type 1 diabetes.

Traditionally, interplay between genetic susceptibility and environmental factors is thought to provide the fundamental element for the disease. The major genetic susceptibility for developing type 1 diabetes is located on the human leukocyte antigen (HLA)-DQ region on chromosome 6. Two HLA-DQ haplotypes, DQA1*0301-DQB1*0302 (DQ8) and DQA1*0501-DQB1*0201 (DQ2) are associated with high risk for developing diabetes. Almost 90% of patients with type 1 diabetes have at least one of these two haplotypes, compared to 20% of the general population (Redondo et al., 2001). These susceptibility genes are important regulators of the immune response; the molecules they encode reside on the cell surface and have the ability to process and present antigens to autoreactive T-cells.

In genetically susceptible individuals there is a pathological presentation of autoantigens on the cell surface of the pancreatic islet cells. This, in combination with a failing negative selection of the T-cells in the thymus and decreased regulatory capacity of regulatory T-cells in peripheral blood (Lindley et al., 2005), leads to an inflammatory response within the pancreatic islets as well as to the production of antibodies against β-cell antigens. Islet cell antibodies were the first ones described, but we now also recognize autoantibodies to insulin, glutamic acid decarboxylase and protein tyrosine phosphatase. Up to 90% of the patients with new onset type 1 diabetes have autoantibodies directed against one or more of these autoantigens It is yet to prove if the autoantibodies have an active role in the pathogenesis of type 1 diabetes but the presence and persistency of autoantibodies appear to increase the likelihood of developing type 1 diabetes. The inflammatory response leads to destruction of the pancreatic β-cells and progressive loss of insulin secretion until reaching critically low levels or even complete insulin deficiency.

3. Vascular endothelium in type 1 diabetes

In children with diabetes risk HLA, signs of systemic endothelial cell activation can be seen already before the clinical onset of diabetes (Toivonen et al, 2004), suggesting it to be an early event in the disease process. Postmortem studies near the onset of type 1 diabetes have shown that class II HLA molecules may be abundantly expressed on vascular endothelial cells lining the capillaries and capillary sinusoids in the islets (Itoh et al., 1993). The upregulation of HLA is paralleled by strong expression of adhesion molecules (i.e. ICAM-1) in the same endothelial areas (Hanninen et al., 1992). These events, seemingly induced by circulating proinflammatory mediators, facilitate homing and migration of inflammatory cells such as T cells across the dysfunctional endothelium . Interaction between antigen-specific T-cells and antigen/HLA complexes on the endothelial cells surface induced a rapid transmigration of the T-cells across the endothelial cell layer (Greening et al., 2003). Similar changes may be found on the surface of endothelial cells of other vascular beds (Greening et al., 2003).This fits well with the clinical observation that type 1 diabetes is often comorbid with chronic autoimmune diseases in other organs such as gut (celiac disease) and thyroid

gland (autoimmune thyroiditis). These disorders also share some of the HLA DQ diabetes risk alleles. An activated endothelium could be the link.

It is well known that long-term exposure to hyperglycemia, hyperlipidemia and inflammation, all of which being important features of type 1 diabetes, is harmful to the endothelial cells, causing further endothelial dysfunction, and in the long run, accelerated atherosclerosis. Lymphocyte accumulation within the arterial wall is an important mechanistic component in the atherosclerotic process and contributes to endothelial cell injury and dysfunction. The endothelial injury, in turn, promotes additional immune events, including release of different chemokines and cytokines resulting in further transmigration of immune cells, and synthesis of C-reactive protein via liver activation by interleukin-6.

Recent study, assessing the relationship of genetic susceptibility with endothelial dysfunction in young patients with type 1 diabetes, found significant correlation between HLA-DQ 2/8, which confers the highest risk for developing type 1 diabetes, and cutaneous microvascular dysfunction (Odermarsky et al., 2007). This could imply, although its does not prove, a role for HLA in the pathogenesis of type 1 diabetes. Further studies at our center are under way to investigate whether such changes could be present already before the clinical onset of type 1 diabetes.

4. Exogenous risk factors and type 1 diabetes: Is vascular endothelium a link?

Putative environmental triggers include viruses, environmental toxins and foods, but it has been difficult to demonstrate a reproducible correlation between them and the development of type 1 diabetes (The environmental determinants of diabetes in the young (TEDDY) Study Group, 2008). Although appealing, there is no evidence to date of a direct interplay between infections and genetics in the causation of type 1 diabetes. the risk of developing T1D seems to increase with the number of infections experienced by an individual during the year preceding the onset of T1D. Although we currently lack the knowledge of the precise underlying mechanisms, there are other reports on similar associations between infectious recurrence and chronic diseases such as multiple sclerosis or rheumatoid arthritis. In some animal studies, the development of atherosclerotic plaque was accelerated by repeated infection. One possible mechanistic link between these chronic inflammatory diseases (e.g. atherosclerosis and T1D) and infection might be endothelial injury. Infections cause vascular endothelial dysfunction, which may persist for up to 1 year after the infectious illness. Mild respiratory infections (“common cold”) seem to aggravate arterial endothelial dysfunction in young patients with T1D. Those with increased recurrence of infections of this type are more susceptible to decreased carotid artery elasticity. The latter was earlier shown to be in part dependent on the functional integrity of endothelial cells. In atherosclerosis-susceptible mice, the degree of endothelial vasomotor dysfunction in skin microcirculation correlates with the number of pathogen inoculations (Odermarsky M, Liuba P unpublished data).

It has been shown that even mild viral infection causes vascular endothelial dysfunction, which may persist for up to 1 year after the infectious illness (Charakida et al., 2005). Infections promote the inflammatory environment needed for endothelial cell activation and upregulation of HLA. These changes could, if genetic susceptibility is present, contribute to homing, transmigration, and accumulation of inflammatory cells in certain tissues. In the

pancreas these microcirculatory changes could perhaps have a role in the pathogenesis of type 1 diabetes, but this is still hypothetical.

5. Endothelial function and dysfunction

The endothelial cells line the inner surface of all blood vessels, providing a metabolically active interface between blood and tissue. These cells modulate blood flow, nutrient delivery, coagulation and thrombosis, and leukocyte diapedesis. The endothelium synthesizes important bioactive substances. Of these, nitric oxide is the most potent vasodilator and protector of vascular function, inhibiting platelet activation and aggregation, preventing leukocyte adhesion and migration through the vessel wall, diminishing smooth muscle cell proliferation and migration, and counteracting adhesion molecule expression (Beckman et al., 2002). Nitric oxide is synthezised by the endothelial isoform of nitric oxide synthase. The process involves enzymatic conversion of L-arginine into nitric oxide and L-citrulline. The release of nitric oxide can be up- or downregulated by different factors. The hormone estrogen, physical exercise, and certain dietary factors are examples of upregulators, whereas smoking and oxidized low-density lipoproteins, via oxidative stress, are examples of downregulators (Michel & Vanhoutte, 2010).

Endothelial dysfunction is considered to be the first step in a long-lasting and complex development that leads to atherosclerosis. The dysfunction is a result of an imbalance in the redox-equilibrium towards oxidative stress leading to impaired nitric oxide bioavailability, either caused by its reduced synthesis or by increased breakdown via reactive oxygen species. (Versari et al., 2009). The dysfunctioning endothelium may produce other substances and mediators such as endothelin 1, thromboxane A_2, prostaglandin H and reactive oxygen species, with vasoconstricting, pro-inflammatory, and proatherosclerotic effects (Virdis et al., 2010).

Given the excess of inflammatory and oxidative stress in type 1 diabetes, the endothelium in individuals with type 1 diabetes is continually exposed to factors promoting the development of endothelial dysfunction. The hyperglycemia, excess free fatty acid release, and insulin resistance leads to adverse events within the endothelial cell (Beckman et al., 2002). Recent findings support the concept that genetically susceptible individuals, i.e. diabetes high-risk HLA, are more prone to develop endothelial dysfunction (Odermarsky et al., 2007) and ongoing studies are investigating whether this dysfunction in fact may precede the clinical onset of type 1 diabetes.

The generalized microvascular dysfunction in type 1 diabetes is an important mechanism in the development of these microvascular complications. Nephropathy, retinopathy and neuropathy are all related to damage to the small vessels of the kidney, retina and nerves. Significant associations have been reported between the different microvascular complications of type 1 diabetes; patients with one complication often develops a second one, suggesting common risk factors and pathogenetic mechanisms (Girach & Vignati, 2006).

Overt microvascular disease is however rare during childhood and adolescence. Early signs, such as increases in albumin excretion rates and glomerular filtration rates, renal hypertrophy, changes in retinal microvasculature and impaired autonomic nervous system function may be detectable in kids with type 1 diabetes and often progress during puberty. Microalbuminuria is the earliest stage of clinical nephropathy and is predictive of progression to overt diabetes nephropathy and, notably, of cardiovascular disease (Rossing et al., 1996).

6. Methods for evaluating endothelial function

The ability to detect endothelial dysfunction, before it progresses to overt vasculopathy, could facilitate the early diagnosis and management of high-risk individuals in childhood. There are several techniques that can be used, though they are not in clinical use to any higher extent. Here we will present two methods; flow-mediated dilatation and laser doppler flowmetry with iontophoresis, which are the ones used in our research.

6.1 Assessment of arterial dysfunction via flow-mediated dilatation of the brachial artery

Blood vessels respond to an increase in blood flow, or more precisely shear stress, by dilating. This phenomenon is called flow-mediated dilatation. The principal mediator for this is endothelium-derived nitric oxide. Assessment of flow-mediated dilatation of the brachial artery safely and non-invasively provides a measure of the systemic endothelial function. The brachial artery response to increased shear stress has been shown to correlate significantly with invasive testing of brachial (Irace et al., 2001) and coronary endothelial function (Andersson et al., 1995), as well as with the extent and severity of coronary atherosclerosis (Neunteufl et al., 1997), and carotid artery intima-media thickness (Gaeta et al., 2000).

Several factors affect the response to the increase in shear stress, including temperature, food, drugs and sympathetic stimuli, female hormonal status, among others, and when conducting a study using this technique you must take these confounding factors into consideration. Ultra sound systems used must be equipped with software for two-dimensional imaging, colour and spectral Doppler, an internal electrocardiogram monitor and a high-frequency vascular transducer. A straight segment of the brachial artery above the antecubital fossa is imaged in the longitudinal plane with the ultrasound probe securely fixed using a stereotactic clamp. A blood pressure cuff is then placed on the forearm and inflated to supra-systolic pressure. After cuff release, reactive hyperaemia results and is quantified using Doppler. The arterial diameter is recorded at end diastole using electrocardiographic gating to determine the response of the brachial artery to increase in flow. The flow-mediated dilatation is expressed as a percentage change of the arterial diameter from the baseline vessel size (Corretti et al., 2002; Thijssen et al., 2011). To control the smooth muscle cells ability to dilate the vessel, independently of the endothelium, and to determine maximum obtainable vasodilatation a dose of nitro-glycerine is administrated via spray or sublingual tablet.

6.2 Assessment of microvascular dysfunction via laser doppler flowmetry with iontophoresis in the skin

Iontophoresis is a non-invasive method of introducing charged substances across the surface of the skin by means of a small electric charge. The basic principle is that molecules of drugs in a solution that are positively or negatively charged will migrate across the skin under influence of an applied current according to the rule that like charges repel each other. The amount of drug delivered is dependent on the magnitude and duration of the current applied. The response in the skin vasculature is measured via a laser doppler device (Morris & Shore, 1996). The coherent light directed at the skin changes when it comes in contact with moving tissues (red blood cells) and the emerged light, i.e. skin perfusion, is measured by a photodiode.

Acetylcholine is the standard test drug for the assessment of endothelial function. The response to acetylcholine, using iontophoresis, correlates with diabetes duration and level of glycosylated haemoglobin (Khan et al., 2000). The mechanisms of acetylcholine-induced vasodilatation via ionphoresis remain debatable. In humans, nitric oxide appears to be the main mediator, but other endothelium-dependent vasodilators may contribute as well (Turner et al., 2008).
Sodium nitroprusside is a nitric oxide donor and acts directly, i.e. endothelium-independent, as a control on the smooth muscle cells causing vasodilatation. Cathodal current is used for delivering sodium nitroprusside.
The current for both substances is set to 100 μA for 20 seconds. For each of the substances to be tested five consecutive and equal doses are applied to generate dose-response curves. Baseline perfusion and changes in response to the substance are expressed as area under the curve.

7. The ”common soil” hypothesis-is this applicable in type 1 diabetes?

In 1995, Michael Stern put forward the “common soil” hypothesis, which suggests a shared genetic and environmental origin for type 2 diabetes and atherosclerosis (Stern, 1995). According to this hypothesis, infections leading to chronic inflammation could pertain to the group of environmental etiological factors. Indeed, the risk of developing type 1 diabetes – a condition associated with significant morbidity in cardiovascular diseases in adult life – could rise during viral infections (Blom et al., 1991). Moreover, the risk for childhood diabetes seems to increase in accordance with a higher number of infections during the year preceding diagnosis. Studies in rodent models of atherosclerosis suggest similar dose-dependent association between infection and vascular changes (Liuba et al., 2000; Tormakangas et al., 2005). Furthermore, diabetic patients are more vulnerable to viral infections due to defective lymphocyte-related immunity. In a previous cross-sectional study on diabetic children, we found that recurrent viral infections in the upper airways (“common cold”) during the past year had cumulative adverse effects on the elastic properties (i.e. compliance) of carotid arteries. In a multivariate analysis, the number of viral infections, along with age and plasma levels of glycosylated hemoglobin, significantly and independently predicted the decrease in carotid artery compliance (Odermarsky et al., 2008a). Although impaired carotid elasticity is generally regarded as a marker of early atherosclerosis, these findings do not necessarily imply causality to accelerated atherosclerosis. Prospective studies in children are currently in progress.

8. Prevention strategies in type 1 diabetes via vascular pathways?

Should vascular endothelial dysfunction prove to play a pivotal role in the pathogenesis of both type 1 diabetes and its associated vascular disease, it is then conceivable that combined endothelium-targeting and immunoregulatory strategies already in diabetes-risk individuals without overt type 1 diabetes (i.e. diabetes high-risk HLA individuals) might reduce not only the cardiovascular burden but also the prevalence of type 1 diabetes later in life. Dietary supplementation with L-arginine (substrate for nitric oxide synthesis via nitric oxide synthase), or antioxidants, in order to improve nitric oxide bioactivity, could for instance be relatively simple, risk-free strategy with potential benefit on endothelial dysfunction. Further studies are needed to provide additional mechanistic insights into the

gene-environment interaction on vascular endothelium and the timing and role of endothelial dysfunction in the development of type 1 diabetes and associated cardiovascular disease.

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8

Cardiovascular Autonomic Dysfunction in Diabetes as a Complication: Cellular and Molecular Mechanisms

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1. Introduction

Diabetes is a major world health problem, which affects more than 23 million people in the US and an estimated 250 million worldwide. Diabetes mellitus is a metabolic disease characterized by high blood glucose levels resulted from an inability in pancreatic insulin secretion or insulin resistance. Usually diabetes mellitus is mainly divided into type 1 diabetes characterized by loss of the insulin production from beta cells of the pancreatic islets and type 2 diabetes characterized by insulin resistance (defective responsiveness of body tissues to insulin) and relatively reduced insulin secretion. Although type II diabetes is by far the most common affecting 90 to 95% of the US diabetic population, the studies focusing on the type 1 diabetes cannot be ignored because about 1 in every 400 to 600 children and adolescents has type 1 diabetes and about 2 million adolescents aged 12-19 have pre-diabetes in the US.

Diabetes mellitus is chronic progressive disease that usually cannot be cured. Following the natural progression of disease, diabetes without proper treatments can cause many severe complications including diabetic ketoacidosis, cardiovascular disease, chronic renal failure, retinal damage. These complications obviously enhance the risk for diabetic patients. In these complications of the diabetes mellitus, cardiovascular autonomic dysfunction is a serious although poorly understood long term diabetic complication. Indeed, diabetic patients with cardiovascular autonomic dysfunction have consistently been shown to have an enhanced risk of premature death (Rosengard-Barlund *et al.*, 2009). More importantly, the age-adjusted relative risk for cardiovascular disease in type 1 diabetes far exceeds that of type 2 diabetes (Krolewski *et al.*, 1987; Libby *et al.*, 2005). Therefore, exploring the mechanisms responsible to the cardiovascular autonomic dysfunction can provide an important and new pharmacological and genetic target for improving the prognosis and reducing the mortality in diabetic state.

2. Baroreflex dysfunction in type 1 diabetes and the contribution of the baroreflex dysfunction in prognosis and mortality of the type 1 diabetes

Cardiovascular autonomic function is the autonomic neural regulation of cardiovascular function, which presents the balance between sympathetic and parasympathetic innervation

resulting in periodic fluctuation in heart rate and rhythm. Although there are many invasive and non-invasive methods to evaluate the cardiovascular autonomic function in diverse clinical and research settings, cardiovascular autonomic function typically is measured by a short-term evoked cardiovascular reflex, especially arterial baroreflex.

2.1 Baroreflex dysfunction

The arterial baroreflex normally acts to prevent wide oscillations in blood pressure and heart rate, acting on both sympathetic and parasympathetic limbs of the cardiovascular autonomic nervous system. Dysfunction of the arterial baroreflex control on the blood pressure and heart rate has been described in many studies not only in the type I diabetic patients, but also in experimental models of the type I diabetes.

In the diabetic patients, heart rate variability is the most widely used index of the arterial baroreflex function. Some studies in more heterogeneous groups of patients with type 1 diabetes have indicated that: (1) showing lower global heart rate variability; (2) relative increase in the low-frequency component (sympathetic activity) of the heart rate variability; (3) relative reduction in the high-frequency component (parasympathetic activity) of the heart rate variability; and (4) higher ratio of the low frequency to the high-frequency (Lishner *et al.*, 1987; Rosengard-Barlund *et al.*, 2009; Ziegler *et al.*, 2001). Clinical research data have confirmed that arterial baroreflex sensitivity is reduced in type 1 diabetic patients with a wide range of age and diabetic duration (Lefrandt *et al.*, 1999; Weston *et al.*, 1996;Weston *et al.*, 1998; Dalla *et al.*, 2007). More importantly, this attenuated arterial baroreflex function was found in the type 1 diabetic patients without the clinical complications, the alterations of the other autonomic function tests, or the overt autonomic neuropathy (Lefrandt *et al.*, 1999; Rosengard-Barlund *et al.*, 2009). Therefore, it is of note that the reduced arterial baroreflex sensitivity can be an earlier sensitive marker of the cardiovascular autonomic dysfunction in the type 1 diabetic patients.

In order to obtain new insights into human type 1 diabetes, animal models of the type 1 diabetes have been widely used in the biomedical studies focusing on the type 1 diabetes, such as alloxan-induced diabetic rabbits (McDowell *et al.*, 1994b), streptozotocin-induced diabetic rats (Hicks *et al.*, 1998; Maeda *et al.*, 1995; Van *et al.*, 1998; Chen *et al.*, 2008), and calmodulin transgenic OVE26 diabetic mice (Gu *et al.*, 2008). Streptozotocin (STZ)-induced diabetic rat is an animal model of insulin-dependent diabetes usually used to study the cardiovascular alterations including cardiovascular autonomic dysfunction caused by diabetes even if the changes of cardiovascular function in this animal model don't fully match the alterations observed under the clinical type 1 diabetic states (Hicks *et al.*, 1998). In the STZ-induced diabetic rats, the arterial baroreflex dysfunction is presented as early as 5 days after the STZ administration (Maeda *et al.*, 1995). Much evidence has documented that the arterial baroreflex is decreased in all kinds of type 1 diabetic models (Chen *et al.*, 2008; Dall'Ago *et al.*, 1997; De Angelis *et al.*, 2000; Gu *et al.*, 2008; Maliszewska-Scislo *et al.*, 2003; McDowell *et al.*, 1994b; Van *et al.*, 1998).

2.2 Association of cardiovascular autonomic dysfunction with mortality rate

30 years ago, Ewing et al (Ewing *et al.*, 1980) first reported that there was a mortality rate of 53% after 5 years in diabetic patients with abnormal autonomic function, compared with a mortality rate of about 15% over the 5 year period among diabetic patients without abnormal autonomic function. Thereafter the growing evidence has confirmed that

cardiovascular autonomic dysfunction is associated with a high risk of cardiac arrhythmias and with sudden death in the diabetic state. A longitudinal study by O'Brien et al (O'Brien *et al.*, 1991) has investigated 5-year survival in 506 randomly selected patients with insulin-dependent diabetes mellitus. In this study, the cumulative 5-year mortality rate in the diabetic patients with cardiovascular autonomic dysfunction (27%) is about 5-fold more than in the diabetic patients with normal cardiovascular autonomic function (5%). However, there is no difference in duration of diabetes between the deceased diabetic patients with and without cardiovascular autonomic dysfunction (O'Brien *et al.*, 1991). A meta-analysis (Maser *et al.*, 2003) and the epidemiology of diabetes complication study (Orchard *et al.*, 1996) also showed that cardiovascular autonomic dysfunction could contribute to the increased risk of mortality rate in the individuals with diabetes. In the recent EURODIAB prospective complications study, the researchers have found that cardiovascular autonomic dysfunction is an important risk marker for mortality rate, exceeding the effect of the traditional risk factors (such as age, waist-to-hip ratio, pulse pressure, and non-HDL cholesterol) (Soedamah-Muthu *et al.*, 2008).

Since the diabetic patients are more likely to have many known diabetes-associated risk factors besides cardiovascular autonomic dysfunction (Soedamah-Muthu *et al.*, 2008), the question is whether cardiovascular autonomic dysfunction is an independent risk factor to predict the mortality rate of the diabetic patients. Some studies have addressed this question to minimize the potential interference of other risk factors (for example age, sex, height, smoking, diabetes duration, etc) by matching these variables in the diabetic patients with and without cardiovascular autonomic dysfunction (O'Brien *et al.*, 1991; Orchard *et al.*, 1996; Rathmann *et al.*, 1993). In Rathmann's study (Rathmann *et al.*, 1993), diabetic patients with and without cardiovascular autonomic dysfunction were matched for age, sex, and duration of diabetes. The 8-year survival rate estimate in patients with cardiovascular autonomic dysfunction was 77% compared with 97% in those with normal cardiovascular autonomic function in this study (Rathmann *et al.*, 1993). O'Brien et al. have also matched age, sex, and duration of diabetes in the diabetic patients with and without cardiac autonomic dysfunction in their study (O'Brien *et al.*, 1991). They found that the cardiovascular autonomic dysfunction was associated with the mortality rate of the type 1 diabetic patients (O'Brien *et al.*, 1991).

2.3 Potential mechanisms responsible for cardiovascular autonomic dysfunction-increased mortality rate

Although many studies mentioned above have confirmed that the cardiovascular autonomic dysfunction is involved in increasing mortality rate of type 1 diabetic patients, we really don't know whether cardiovascular autonomic dysfunction is directly or indirectly responsible for the increased mortality rate. It is possible that several possible mechanisms are involved in this clinical phenomenon.

First, a few clinical studies have reported that some type 1 diabetic patients in good health the previous day are found dead in the morning in an undisturbed bed with no sign of the symptoms (such as sweating and struggle) and negative autopsy results, which is named as the 'dead in bed' syndrome (Tattersall & Gill, 1991; Weston & Gill, 1999). One recent clinical study has found that ECG abnormalities including QT prolongation, cardiac rhythm disturbance, and subsequent ventricular tachyarrhythmia appear in the ambulant patients with type 1 diabetes (Gill *et al.*, 2009). The ECG abnormalities can serve as

principal underlying causes of the 'dead in bed' syndrome (Gill *et al.*, 2009). Cardiovascular autonomic dysfunction itself can link to the QT prolongation and sudden death (Weston & Gill, 1999). In another study, type 1 diabetic adolescents with impaired cardiovascular autonomic function are associated with the possible development of cardiac arrhythmias and left-ventricular hypertrophy (Karavanaki *et al.*, 2007). In addition, decreased heart rate variability is also a predictive risk factor for ventricular arrhythmia and sudden cardiac death (Kleiger *et al.*, 1987). Loss of cardiac vagal drive combined with loss of baroreceptor reflex sensitivity is thought to mediate the decreased heart rate variability and autonomic instability that exacerbate arrhythmia susceptibility (Binkley *et al.*, 1991). These studies indicate that cardiovascular autonomic dysfunction (decreased heart rate variability and loss of baroreceptor reflex sensitivity) is correlated with the prognosis and mortality in patients with type 1 diabetes via increasing the susceptibility to the lethal arrhythmias.

Second, although cardiovascular autonomic dysfunction is an independent risk factor to predict the mortality rate of the diabetic patients described above, other abnormalities (such as increased stiffness of the vascular walls at the site of the arterial baroreceptors, left ventricular hypertrophy, endothelial dysfunction, renal failure, peripheral neuropathy, etc) usually coexist with cardiovascular autonomic dysfunction in type 1 diabetic patients (Toyry *et al.*, 1997; Lluch *et al.*, 1998; Lefrandt *et al.*, 2010). Therefore, it is possible that the interaction between cardiovascular autonomic dysfunction and other concomitant abnormalities is responsible for the increased mortality rate in type 1 diabetic patients. It has been shown that cardiovascular autonomic function is easily impaired in type 1 diabetic patients with microalbuminuria (renal dysfunction) (Lefrandt *et al.*, 1999; Clarke *et al.*, 1999). O'Brien et al have reported that renal failure-induced mortality rate is higher in type 1 diabetic patients with cardiovascular autonomic dysfunction than in those without cardiovascular autonomic dysfunction (O'Brien *et al.*, 1991). In a 23 year follow-up study, cardiovascular autonomic dysfunction may be involved in a higher mortality rate induced by microalbuminuria in type 1 diabetic patients (Messent *et al.*, 1992). Similarly, renal disease also can partially explicate the cardiovascular autonomic dysfunction-increased mortality rate in patients with diabetes mellitus (Weinrauch *et al.*, 1998; Kim *et al.*, 2009). In addition, using logistic regression analysis, one recent study has addressed the relationship between cardiovascular autonomic dysfunction and other abnormalities in 684 type 1 diabetic patients (Pavy-Le *et al.*, 2010). The research data have shown that retinopathy, peripheral neuropathy, and erectile dysfunction are closely correlated to the severity of the cardiovascular autonomic dysfunction (Pavy-Le *et al.*, 2010). Furthermore, some studies have also found a consistent association between cardiovascular autonomic dysfunction and silent myocardial ischemia, in which the patient's risk coefficient related to the cardiovascular autonomic dysfunction is higher in asymptomatic diabetic patients with silent myocardial ischemia than in those without silent myocardial ischemia (Valensi *et al.*, 2001; Vinik & Ziegler, 2007; Katz *et al.*, 1999).

Finally, several studies reported the involvement of cardiorespiratory arrest in the mortality of the diabetic patients with cardiovascular autonomic dysfunction (Page & Watkins, 1978; Bergner & Goldberger, 2010; Douglas *et al.*, 1981). The research data from Page et al. (Page & Watkins, 1978) have demonstrated that young diabetic patients with severe cardiovascular autonomic dysfunction can appear to have cardiorespiratory arrest due to the impairment of cardiorespiratory function. The cardiorespiratory arrest may be responsible for the mortality of these diabetic patients (Page & Watkins, 1978).

3. Mechanisms responsible for the reduced baroreflex function in type 1 diabetes

The arterial baroreflex is a homeostatic mechanism that alters heart rate and blood pressure in response to changes in arterial wall tension detected by the baroreceptors in the carotid sinus and aortic arch. The arterial baroreflex arc includes an afferent limb, a central neural component and autonomic neuroeffector components. As the primary afferent limb of the baroreceptor reflex, baroreceptor neurons sense blood pressure by increasing their discharge (excitation) when arterial blood pressure rises. This excited signal in baroreceptor neurons reaches to the dorsal medial nucleus tractus solitarii (NTS, the first site of baroreceptor neuron contacting with central nervous system), in which the integrated input signal inhibits the efferent sympathetic outflow to the heart and peripheral vascular, and activates efferent parasympathetic activity to the heart those decrease peripheral vascular resistance, heart rate, and arterial blood pressure. Conversely, the baroreceptor afferent signal decreases when arterial blood pressure falls, which reflexly induces an increase in heart rate and arterial blood pressure.

As mentioned above, blunted arterial baroreflex sensitivity is observed in type 1 diabetic patients and animal models. What are the mechanisms responsible for the attenuated arterial baroreflex sensitivity in type 1 diabetes? Every site within the baroreflex arc may be responsible for the depressed baroreflex sensitivity in type 1 diabetes. Therefore, we will discuss the fact that the reduced baroreflex sensitivity results from functional and/or structural changes in the baroreceptors (including nerve terminals and neuron somata), central neural integration, and autonomic efferent component.

3.1 Role of baroreceptor in the blunted arterial baroreflex in type 1 diabetes

As the primary afferent limb of the arterial baroreceptor reflex, baroreceptor neurons are pseudo-unipolar neurons (T-shaped neurons) consisting of a cell body existing in the nodose or petrosal ganglia and an initial axon segment. This axon segment bifurcates near the soma into a peripheral process innervating aortic arch and carotid sinus for sensing the alteration of the arterial blood pressure and a central process terminating in the NTS for conveying the afferent signals to the central nervous system. The mechanisms responsible for mediating afferent sensitivity of barosensitive neurons to pressure are complex and not thoroughly understood. The process of translating changes in arterial wall tension into impulse traffic to the NTS involves 2 broad functional steps: 1) mechanotransduction which is governed by the properties of mechanosensitive ion channels in the nerve terminal and the mechanical properties of the coupling of the arterial wall to the sensory terminal; and 2) spike initiation which is governed by the excitability of membrane voltage sensitive ion channels that influence the electrical (cable) properties of the axonal process and cell body. All of these factors could be (and likely are) altered in type 1 diabetes, which can directly affect the arterial baroreflex function.

3.1.1 Changes of baroreceptor afferent nerve and terminal in type 1 diabetes

Although some studies have suggested that diabetes-induced postural hypotension results from impairments of afferent baroreceptors and of sympathetic neurons innervating the vascular wall and heart in diabetic patients (Low *et al.*, 1975; Iovino *et al.*, 2011), there is only fragmentary evidence to support this assumption because of the inability of clinical cardiovascular autonomic function tests to separate the role of the afferent, central, and

efferent components of the arterial baroreflex. In general, the function of the baroreceptor afferent nerve and terminal is investigated by recording the single fiber or multifiber activity of the aortic depressor nerve or carotid sinus nerve in a perfused isolated aortic arch/carotid sinus preparation (do Carmo *et al.*, 2007; Doan *et al.*, 2004; Fazan, Jr. *et al.*, 1997; McDowell *et al.*, 1994b; Reynolds *et al.*, 1994; Reynolds *et al.*, 1999; Xiao *et al.*, 2007; Zhang *et al.*, 2004). However, the baroreceptor function in the diabetic state is studied only in the aortic depressor nerve (Fazan, Jr. *et al.*, 1997; Fazan, Jr. *et al.*, 1999; McDowell *et al.*, 1994b; Reynolds *et al.*, 1999) but not in the carotid sinus nerve (Salgado *et al.*, 2001). This may be because there are only baroreceptor afferent fibers and no chemoreceptor afferent fibers in rat aortic depressor nerve unlike the carotid sinus nerve (Fan *et al.*, 1996; Kobayashi *et al.*, 1999; Sapru & Krieger, 1977; Sapru *et al.*, 1981). Based on the results from some studies, there is no evidence to show the changes of the aortic depressor nerve activity in STZ-induced type 1 diabetic rats (Fazan, Jr. *et al.*, 1997; Reynolds *et al.*, 1999; Dall'Ago *et al.*, 2002) and alloxan-induced diabetic rabbits (McDowell *et al.*, 1994b), compared to the sham animals. In addition, Gu *et al* (Gu *et al.*, 2008) have found that the baroreceptor function of the aortic depressor nerve is preserved in the ascending phase of the arterial blood pressure but is blunted in the descending phase of the arterial blood pressure in type 1 diabetic mice. Nevertheless, the results obtained by a new approach, named as cross-spectral analysis, indicate that a significant decrease of the aortic baroreceptor nerve function is observed in anesthetized rats with either short term (10-20 days) or long term (12-18 weeks) STZ-induced diabetes (Fazan, Jr. *et al.*, 1999). This new approach uses the magnitude of the transfer function obtained by analyzing the relationship between beat-by-beat time series of mean arterial blood pressure and aortic depressor nerve activity as the index of the aortic baroreceptor nerve function, whose advantage is to evaluate the aortic baroreceptor nerve function under more physiological conditions (Salgado *et al.*, 2001; Fazan, Jr. *et al.*, 1999; deBoer *et al.*, 1987) compared to the arterial blood pressure/aortic depressor nerve activity curve used in other studies (Dall'Ago *et al.*, 2002; Fazan, Jr. *et al.*, 1997; Gu *et al.*, 2008; McDowell *et al.*, 1994b; Reynolds *et al.*, 1999). In addition, Fazan et al have found that the morphological change in the aortic depressor nerve, an afferent arm of the baroreflex may result in the arterial baroreflex impairment in the STZ-induced diabetic rats (Fazan *et al.*, 2006). Therefore, the functional and structural alterations of the baroreceptor afferent nerve in type 1 diabetes still need to be further clarified in future study.

By light, electron, and confocal microscopies, some researchers have identified the aortic baroreceptor terminals in the adventitia of the aortic arch from dogs, rabbits, cats, rats, and mice (Aumonier, 1972; Cheng *et al.*, 1997; Krauhs, 1979; Li *et al.*, 2010). More importantly, Li et al have demonstrated that diabetes induces morphological atrophy of the aortic baroreceptor terminals in type 1 diabetic mice (Li *et al.*, 2010). However, there is no report on the functional role of the aortic baroreceptor terminals in sham and type 1 diabetic animals because it is difficult to separate aortic baroreceptor terminals to other tissues (such as smooth muscle and endothelium) in the aortic arch. It is possible that using gene and short hairpin RNA (shRNA) transfection can solve this problem in future study.

3.1.2 Role of aortic baroreceptor neurons in the arterial baroreflex in the type 1 diabetes

Many studies have used the responses of blood pressure and heart rate to electrical stimulation of baroreceptor-containing nerve (aortic depressor nerve) for the evaluation of the baroreflex sensitivity in rats (Fan & Andresen, 1998; Salgado *et al.*, 2007; Tang &

Dworkin, 2007). The aortic depressor nerves (the peripheral process of the aortic baroreceptor neuron) are composed of both afferent A-type (myelinated) axons (about 25%) and C-type (unmyelinated) axons (about 75%) (Yamasaki *et al.*, 2004). There are very different dynamic sensory discharge characteristics between A-type and C-type baroreceptor afferents. C-type afferents are activated mainly at very high pressure and have lower firing frequencies, irregular discharge patterns (Thoren *et al.*, 1999), and appear to be the primary regulators of tonic baseline levels of arterial blood pressure besides regulating the baroreflex sensitivity (Seagard *et al.*, 1993). A-type afferents have lower pressure thresholds with very stable, proportional firing patterns (Thoren *et al.*, 1999), which are thought to regulate the baroreflex sensitivity but not baseline levels of arterial blood pressure (Seagard *et al.*, 1993). Electrical Stimulation of the rat aortic depressor nerve has several advantages to examine the baroreflex function. First, the rat aortic depressor nerve contains only baroreceptor afferent fibers and no chemoreceptor afferent fibers to transmit the chemoreceptor information (Fan *et al.*, 1996; Kobayashi *et al.*, 1999; Sapru & Krieger, 1977; Sapru *et al.*, 1981). Second, the baroreflex induced by stimulating rat aortic depressor nerve is measured without the aortic baroreceptor terminals in the reflex arc, which allows us to specifically examine the role of electrical excitability of aortic baroreceptor in the baroreflex function (second process mentioned above). Third, by varying the frequency of stimulus, one can differentially activate A- and C- afferent fibers, and thus evaluate the relative contribution of each to the altered aortic baroreceptor excitability and baroreflex function in STZ-induced diabetes. In our preliminary study, the baroreflex responses of blood pressure and heart rate to the electrical stimulation of the aortic baroreceptor nerve are significantly depressed in STZ-induced diabetic rats (Fig. 1). In addition, our study also found that microinjection of angiotensin II type 1 (AT_1) receptor antagonist (20 μM L158,809) into the nodose ganglia significantly improved the baroreflex sensitivity induced by aortic depressor nerve stimulation in STZ-induced diabetic rats (Fig. 1). Simultaneously, AT_1 receptor antagonist also normalized the depressed cell excitability in the aortic baroreceptor neurons of STZ-induced diabetic rats (Li & Zheng, 2011). The fact is that nodose neurons are found to influence the conduction and frequency of the electrical impulses in the baroreceptor central axons projecting to the central nervous system when electrical signals in the baroreceptor peripheral axons reach the nodose neurons (Ducreux *et al.*, 1993). One review paper has concluded that the excitability of vagal afferent neurons has dramatic consequences for the regulation and modulation of vago-vagal reflex (Browning, 2003). Furthermore, Devor (Devor, 1999) has reported that electrical excitability of the soma in the dorsal root ganglia may be required to insure the reliable afferent electrical impulses transmitted to the spinal cord. These results, taken together, demonstrate that the reduced cell excitability of the aortic baroreceptor neurons contributes to the blunted baroreflex sensitivity in STZ-induced diabetic rats.

However, results from reflex experiments evoked by the electrical stimulation need to be tempered because the electrical stimulation technique does not represent a physiological substrate for baroreceptor activation. Thus, arterial baroreflex evoked by changes in arterial blood pressure should be done to further address the role of the aortic baroreceptor neurons in the arterial baroreflex in the type 1 diabetes. Of course, in this approach (blood pressure-mediated baroreflex sensitivity), possible alterations in the mechanotransduction process at the baro-sensory nerve terminal may also play a role in the suppressed baroreceptor function in response to pressure changes.

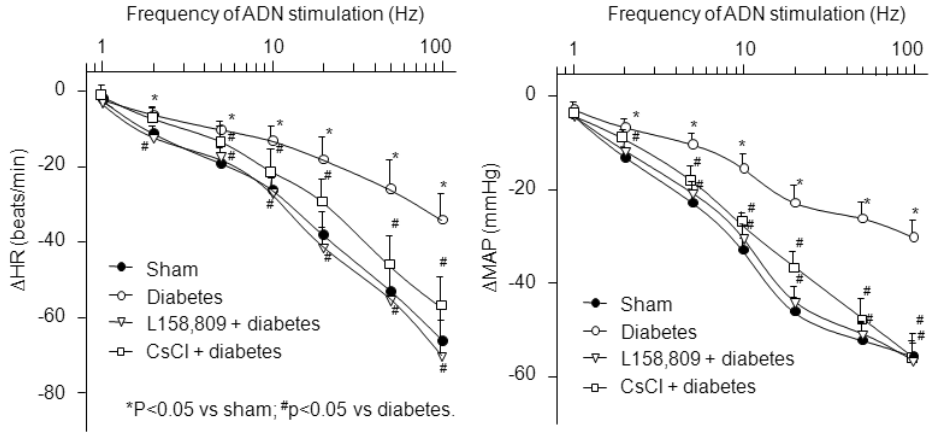

Fig. 1. Reflex ΔMAP and ΔHR in response to different frequencies of ADN stimulation in anesthetized sham and STZ-induced diabetic rats (n=8 in each group). L158,809: AT_1 receptor antagonist; CsCl: HCN channel blocker. MBP, mean blood pressure; HR, heart rate; ADN, aortic depressor nerve.

3.1.3 Contribution of the HCN channel to the cell excitability of aortic baroreceptor neuron in the type 1 diabetes

Until now it is controversial whether either severe degenerative changes or neuronal cell loss in sensory and autonomic nervous tissues are found in STZ-induced diabetic animals (Yagihashi, 1997). Apoptotic cell death was reported in the sensory neurons, satellite cells, and Schwann cells from dorsal root ganglia (DRG) of STZ-induced diabetic rats (Russell *et al.*, 1999; Srinivasan *et al.*, 2000). Kogawa et al. also found that apoptotic cell death of DRG neurons and impaired sensory nerve regeneration were induced by sciatic nerve crush in STZ-induced diabetic rats (Kogawa *et al.*, 2000). On the other hand, the findings from Sango et al (Sango *et al.*, 1991; Sango *et al.*, 1995; Sango *et al.*, 1997) indicated no difference in the dissociated neurons from DRG between sham and STZ-induced diabetic mice. Furthermore, some studies have demonstrated that there are no morphological changes of the peripheral nerves (Sharma & Thomas, 1987) and cell death of the nodose afferent neurons (Sango *et al.*, 2002) in STZ-induced diabetic animals. Our recent study (Tu *et al.*, 2010) also suggests that STZ-induced diabetes does not change the total cell number of the nodose afferent neurons and the ratio of A-/C-type neurons (Fig. 2). These results provide an important piece of information that the parasympathetic reflex dysfunction (Li *et al.*, 2008b; Thomas & Tomlinson, 1993; Ziegler, 1994) in STZ-induced diabetes might be not due to the structural changes in the nodose afferent neurons but most likely due to the functional changes at the cellular and molecular levels.

As everyone knows, many ion channels (such as sodium channels, calcium channels, potassium channels, etc) are responsible for the cell excitation in the excitable cells such as cardiac/skeletal myocytes and neurons including aortic baroreceptor neurons. However, much evidence has indicates that Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels play an important role in the cell excitability of the aortic baroreceptor neurons from sham and STZ-induced diabetic rats.

HCN channels have been found in various types of cells including cardiac and neuronal cells (DiFrancesco, 1985; DiFrancesco, 1993; Pape, 1996). In these spontaneously exciting cells, HCN channels normally associate to the cardiac pacemaker activity and the neuronal oscillatory behavior (Brown *et al.*, 1979; DiFrancesco, 1993; Kaupp & Seifert, 2001; Notomi & Shigemoto, 2004; Pape, 1996; Robinson & Siegelbaum, 2003). However, HCN channels may have a different role in the nodose ganglion neurons (the non-oscillatory and non-automatic exciting cells) because the nodose neurons are inactive except in response to a depolarizing stimulus generated by their peripheral sensory terminals (Doan & Kunze, 1999; Li *et al.*, 2008a). In the nodose neurons, the resting membrane potential is about -50 to -65 mV, in which voltage-dependent sodium, calcium, and potassium channels are almost inactivated (Robinson & Siegelbaum, 2003). The inactivation of these voltage-dependent channels can be recovered to the activation state during the hyperpolarization of the resting membrane potential, which means the number of available voltage-dependent channels for activation is increased if the nodose neurons receive the depolarizing stimulus (Doan & Kunze, 1999). Inhibition of HCN channels has been shown to hyperpolarize the nodose neurons (increasing the resting membrane potential) and to reduce action potential threshold in response to a depolarizing current stimulation, which suggests that HCN channels are involved in the cell excitability of the nodose neurons (Doan *et al.*, 2004; Li *et al.*, 2008a). Results from our recent studies (Li *et al.*, 2008a; Li & Zheng, 2011; Tu *et al.*, 2010) confirm that the HCN current density in A- and C-type aortic baroreceptor neurons from STZ-induced diabetic rats is larger than that from the sham rats (Fig. 3). In addition, the resting membrane potential is depolarized and the current threshold induced the action potentials was elevated in the A-/C-type aortic baroreceptor neurons from STZ-induced diabetic rats, compared with that in sham rats (Li *et al.*, 2008a; Li & Zheng, 2011). Furthermore, HCN channel blockers (CsCl and ZD-7288) lowered the HCN current density, hyperpolarized the resting membrane potential, and raised the cell membrane excitability in A-/C-type aortic baroreceptor neurons from sham and STZ-induced diabetic rats (Li *et al.*, 2008a; Zhang *et al.*, 2010). These results clearly indicate that the HCN channels are involved in the regulation of aortic baroreceptor neuron excitability. The enhancement of HCN currents can contribute to the blunted aortic baroreceptor neuron excitability, and subsequently attenuate the arterial baroreflex sensitivity in STZ-induced diabetic rats. This is true because microinjection of HCN channel blocker (5 mM CsCl) improves the arterial baroreflex sensitivity induced by the electrical stimulation of the aortic depressor nerve (Fig. 1) (Li *et al.*, 2008b).

Four mammalian genes encoding HCN channel isoforms (HCN1, HCN2, HCN3, and HCN4) have been identified (Doan *et al.*, 2004; Ishii *et al.*, 1999; Ludwig *et al.*, 1998; Santoro *et al.*, 1998; Vaccari *et al.*, 1999). In cell lines transfected HCN isoform cDNA, electrophysiological studies have shown that each channel isoform is activated by membrane hyperpolarization with distinct activation kinetics (Ludwig *et al.*, 1999; Moosmang *et al.*, 2001; Qu *et al.*, 2002; Santoro *et al.*, 1998). Activation of the HCN channels is also directly modulated by cAMP, which is dependent on the HCN channel isoform (Stieber *et al.*, 2003; Wainger *et al.*, 2001; Wang *et al.*, 2002). HCN channels are activated with the different activation rates in this order: HCN1>HCN2>HCN3>HCN4 (Accili *et al.*, 2002; Altomare *et al.*, 2001; Moosmang *et al.*, 2001; Stieber *et al.*, 2003; Stieber *et al.*, 2005). HCN1 and HCN3 are only weakly affected by cAMP whereas HCN2 and HCN4 are very sensitive to cAMP (Accili *et al.*, 2002; Stieber *et al.*, 2005; Wahl-Schott & Biel, 2009; Wang *et al.*, 2001). Our studies (Li *et al.*, 2008a; Tu *et al.*, 2010) have found that a fast-activated and cAMP-

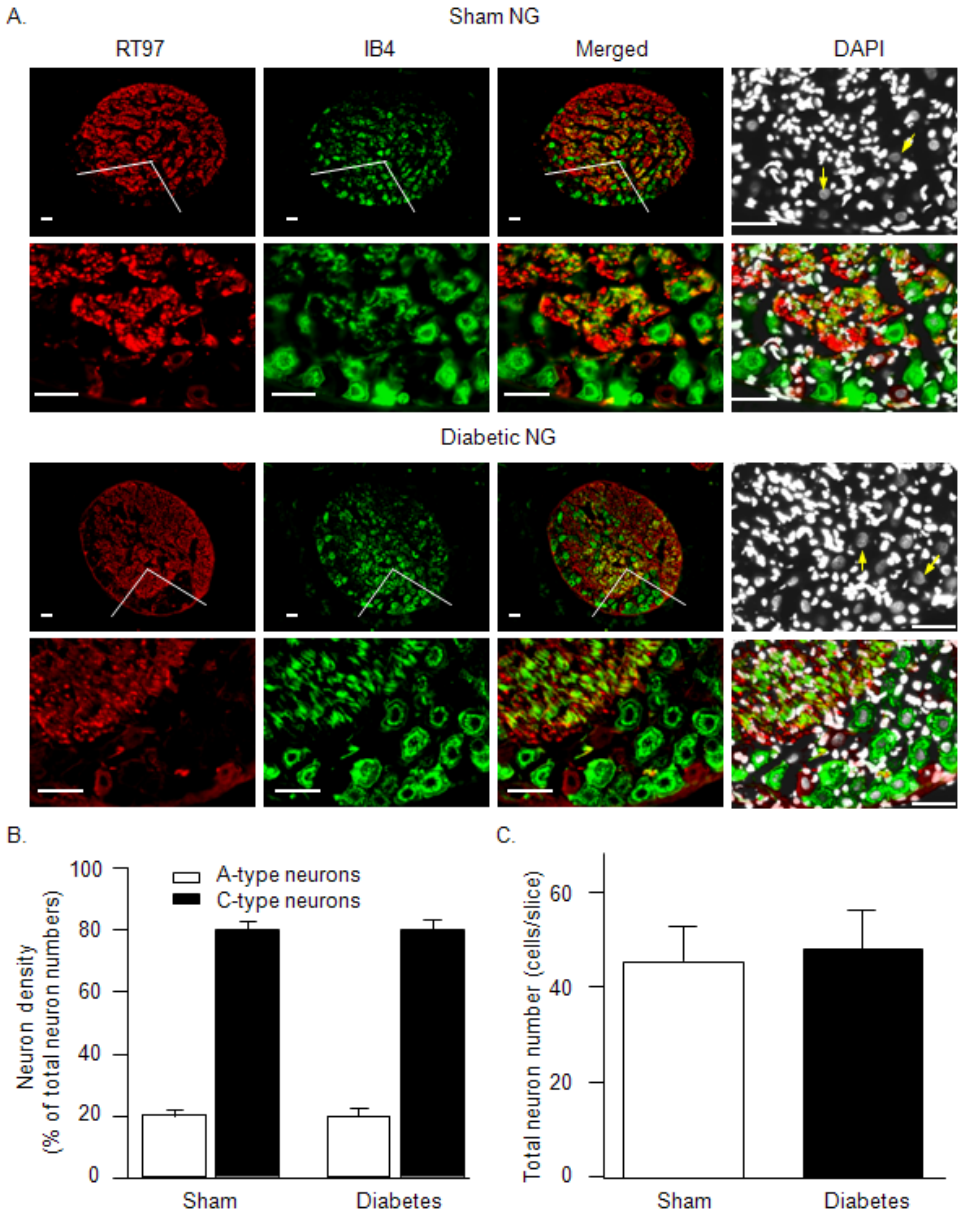

Fig. 2. Ratio of A-type/C-type neurons (A and B) and total neuron number (C) in nodose ganglia from sham and STZ-induced diabetic rats. Calibration bar: 100 μm. RT97, A-type neuron marker; IB4, C-type neuron marker; DAPI, cell nucleus marker. Yellow arrows indicate nodose neurons in DAPI staining (Adapted and reprinted from Tu *et al*., 2010, page 42, with permission from Elsevier)

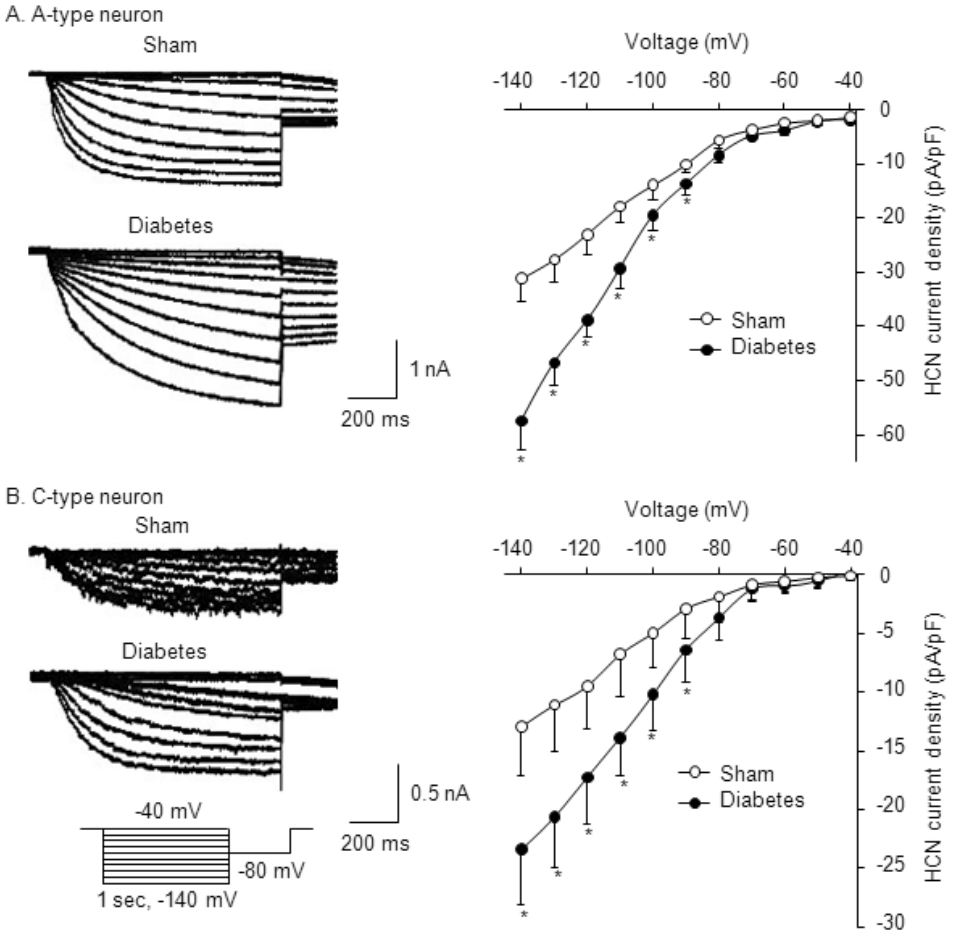

Fig. 3. Original HCN current recording and current density-voltage curves in A- and C-type neurons from sham and STZ-induced diabetic rats. *P<0.05 vs. sham rats (Reprinted from Tu *et al*., 2010, page 48, with permission from Elsevier).

insensitive HCN current is induced in sham A-type aortic baroreceptor neurons whereas a slow-activated and cAMP-sensitive HCN current is induced in sham C-type aortic baroreceptor neurons. From these electrophysiological results, we can imagine that there is a differential distribution of the HCN channel isoforms in the A- and C-type aortic baroreceptor neurons. Data from immunofluorescent double staining also show that HCN1, HCN3, and HCN4 are expressed in sham A-type nodose neurons, whereas HCN2, HCN3, and HCN4 are expressed in sham C-type nodose neurons (Li *et al*., 2008a; Tu *et al*., 2010). Based on these results, it is reasonable to assume that there are marked different activation kinetics and cAMP sensitivity of HCN channels between A-fiber neurons and C-fiber neurons, which might be due to the neuron cell-specific expression of HCN channel isoforms.

Our studies further demonstrate that diabetes enhances the HCN currents and the expression of HCN1, HCN2, and HCH 3 channel proteins in A-type aortic baroreceptor neurons (Li *et al.*, 2008a; Tu *et al.*, 2010). Overexpression of HCN1, HCN2, and HCN3 but not HCN4 channel isoforms can link to the enhanced HCN currents, the slow-activated HCN channel kinetics, and the increased cAMP-sensitivity of HCN channels in diabetic A-type aortic baroreceptor neurons (Li *et al.*, 2008a; Tu *et al.*, 2010). Although diabetes also increases the HCN currents and the expression of HCN2 and HCN3 channel proteins in C-type aortic baroreceptor neurons, diabetes does not change the activation kinetics and the cAMP sensitivity of the HCN channels in C-type aortic baroreceptor neurons due to no expression of HCN1 channel in diabetic C-type aortic baroreceptor neurons (Li *et al.*, 2008a; Tu *et al.*, 2010). From these results, we propose that HCN currents are markedly enhanced via increasing the numbers of HCN channels and sensitivity of HCN channels to cAMP in the aortic baroreceptor neurons. The enhanced HCN currents can contribute to the depressed neuron excitability in diabetic aortic baroreceptor neurons. However, we do realize that these data cannot explain why diabetes induces the different changes of HCN channel protein expression and cannot identify the contribution of the various HCN channel isoforms to the enhanced HCN currents in diabetic A- and C-type aortic baroreceptor neurons.

3.1.4 Regulation of the angiotensin II-superoxide signaling on the HCN channel in the type 1 diabetes

Angiotensin II, an endogenous peptide, has been thought to be a prime candidate in the regulation of the HCN channel function and cell excitability in the diabetic state. It is known that circulating and tissue angiotensin II concentrations are elevated in human and animals with diabetes (Frustaci *et al.*, 2000; Sechi *et al.*, 1994; Shimoni & Liu, 2004). Previous autoradiographic study has identified a high density of angiotensin II receptor binding sites over the nodose neurons (Allen *et al.*, 1988). Widdop, et al. provided evidence for the direct neuronal effects of angiotensin II on the vagal afferent neurons (Widdop *et al.*, 1992). Indeed, our research data not only confirm AT_1 and AT_2 receptors exist in nodose neuronal cells, but also indicate that exogenous angiotensin II enhances the HCN currents and subsequently reduces cell excitability in the aortic baroreceptor neurons from normal rats (Zhang *et al.*, 2010). This is via NADPH oxidase-derived superoxide because a specific HCN channel blocker blunts the inhibitory effect of the exogenous angiotensin II on action potentials (Zhang *et al.*, 2010). More importantly, angiotensin II concentration and protein expression of AT_1 receptors are increased in the nodose neuronal cells from STZ-induced diabetic rats (Li & Zheng, 2011). At the same time, mRNA expression of AT_1 receptors measured by single cell real-time PCR technique is enhanced in the aortic baroreceptor neuron cells from the STZ-induced diabetic rats (Li & Zheng, 2011). In addition, AT_1 receptor antagonist (losartan) significantly normalizes the enhanced HCN currents and the attenuated cell excitability (including depolarization of the resting membrane potential, fall in the input resistance, and decrease in the action potential number) in the aortic baroreceptor neurons induced by diabetes (Li & Zheng, 2011) or exogenous angiotensin II (Zhang *et al.*, 2010). Furthermore, angiotensin II-AT_1 receptor is also involved in the attenuated arterial baroreflex sensitivity in STZ-induced diabetic rats (Fig. 1) (Li *et al.*, 2008b). Based on these results, it is reasonable to assume that elevation of local angiotensin II level can blunt the membrane excitability of the aortic baroreceptor neurons via enhancement of the HCN currents, and consequently attenuate the aortic baroreflex function in the type 1 diabetes.

Above results suggest that elevation of local tissue angiotensin II plays an important role on the enhanced HCN channel activity and the blunted cell excitability in the AB neurons in diabetes. However, it is unclear how angiotensin II and its antagonist within an isolated aortic baroreceptor neuron from diabetic rat interact with AT_1 receptor to affect the HCN channel activity and cell excitability. Classical viewpoint about the effects of angiotensin II binding with AT_1 receptor is that angiotensin II binds with AT_1 receptor at the cell membrane, and following the phosphorylation of the AT_1 receptor, angiotensin II induces intracellular responses via activating intracellular downstream signal transduction. However, Zhuo, et al. (Zhuo *et al.*, 2002) have found that there is substantial intracellular accumulation of angiotensin II in renal cortical endosomes during angiotensin II-dependent hypertension via an AT_1 receptor-mediated process. Recent studies have shown that intracellular administration of angiotensin II increases the peak inward calcium current density and decreases the junctional conductance via intracellular angiotensin II receptors in cardiac myocytes (De Mello, 2003; De Mello & Monterrubio, 2004). Intracellular treatment of losartan (a selective AT_1 receptor antagonist) abolishes the effect of intracellular angiotensin II (Allen *et al.*, 1988; Bacal & Kunze, 1994). Based on these studies, we reason that diabetes-induced elevation of intracellular angiotensin II concentration in the nodose neurons contributes to the enhanced HCN channel activity and the blunted cell excitability in the AB neurons in diabetes. This viewpoint is confirmed by our observation that intracellular administration of losartan (added to the recording pipette solution) decreased the HCN current density and increased the cell excitability in the AB neurons from diabetic rats (Li & Zheng, 2011). Therefore, it is possible there is an intracellular angiotensin II production system in the nodose ganglion tissue. Of course, it would be optimal to measure intracellular angiotensin II concentration in the aortic baroreceptor neurons, but there is no appropriate measurement for it so far due to insufficient cellular material of tiny nodose ganglia. This issue needs to be confirmed by further study.

Growing evidence has shown that the AT_1 and AT_2 receptors are defined on the basis of their opposite pharmacological and biochemical effects (Levy, 2004). Activation of AT_1 receptors mainly results in vasoconstriction, augmentation of cardiac contractility, cell proliferation, vascular and cardiac hypertrophy, oxidative stress, and inhibition of the neuronal potassium currents (Gelband *et al.*, 1999; Levy, 2004; Sumners *et al.*, 1996). On the other hand, stimulation of AT_2 receptors induces vasodilation, anti-growth, anti-hypertrophy, and enhancement of the neuronal potassium currents (Horiuchi *et al.*, 1999; Kang *et al.*, 1995; Martens *et al.*, 1996; Matsubara, 1998; Siragy, 2000). Although AT_2 receptors are expressed in the rat nodose neurons, activation of AT_2 receptors does not affect the activation of HCN channels because AT_2 receptor antagonist (PD123,319) does not alter the effect of angiotensin II on the HCN currents (Zhang *et al.*, 2010). Until now there is no study to explain this result, but it is possible that many factors (such as species, tissue, channel sensitivity, etc) are responsible for this discrepancy.

Now the question is how angiotensin II regulates the activation of HCN channels and what is the downstream of angiotensin II-AT_1 receptor. NADPH oxidase has been considered as a main source of intracellular superoxide in many tissues (Cifuentes *et al.*, 2000; Franco *et al.*, 2003; Gao *et al.*, 2004; Griendling *et al.*, 2000; Li *et al.*, 2007; Schieffer *et al.*, 2000). NADPH oxidase is a multicomponent enzyme composed of three cytosolic subunits ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$), two membrane-associated subunits ($gp91^{phox}$ and $p22^{phox}$), and the small G-proteins (Rac and Rap1a) (Kim & Iwao, 2000; Lassegue & Clempus, 2003). Angiotensin II significantly activates NADPH oxidase via AT_1 receptors, resulting in the superoxide

production (Touyz & Berry, 2002). In the nodose ganglia from STZ-induced diabetic rats, the protein expression of the NADPH oxidase components (gp91^{phox}, p22^{phox}, p40^{phox}, p47^{phox}, and p67^{phox}) is elevated, compared to sham rats (Li & Zheng, 2011). In addition, NADPH oxidase inhibitor or superoxide scavenger significantly improves the superoxide over-production, the enhanced HCN currents, and the lowered membrane excitability induced by exogenous angiotensin II (Zhang *et al.*, 2010) or diabetes (Li & Zheng, 2011). These results strongly indicate that NADPH-derived superoxide can mediate the effect of endogenous angiotensin II on the HCN channels and membrane excitability in diabetic rat aortic baroreceptor neurons.

3.1.5 Role of other channels in the aortic baroreceptor neuron in the type 1 diabetes

Using patch-clamp technique, all major voltage-gated ion channels including channels subunits are recorded in the nodose neurons, such as sodium channels (tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels), calcium channels (N-type, L-type, T-/R-type, and other type calcium channels), and potassium channels (4-aminopyridine-sensitive, tetraethylammonium-sensitive, and calcium-activated potassium channels) (Lancaster *et al.*, 2002; Li *et al.*, 2005; Li & Schild, 2006; Schild & Li, 2001). These channels all are involved in the initiation and formation of the action potential and affect the nodose neuron excitability. Angiotensin II is known to modulate the calcium channel kinetics in the nodose neurons (Bacal & Kunze, 1994; Moreira *et al.*, 2005). However, until now we did not obtain any information about the changes of these channels in the aortic baroreceptor neurons in the type 1 diabetes. Therefore, the role of these channels in the diabetic baroreceptor neurons remains to be revealed.

3.2 Involvement of the central neural component in the blunted arterial baroreflex in type 1 diabetes

Central neural integration of the input signals from the baroreceptors usually occurs at the level of nucleus tractus solitarii and rostral ventrolateral medulla (Spyer *et al.*, 1997). Although many studies have shown that diabetes causes a variety of functional and morphological disorders in the central nervous system (including hippocampus, cortex, and cerebellum) (Biessels *et al.*, 1999; Selvarajah & Tesfaye, 2006; Mooradian, 1997a; Mooradian, 1997b; Guven *et al.*, 2009), the role of the central neural component in the blunted arterial baroreflex in type 1 diabetes is less well documented. One recent study from Gu, et al (Gu *et al.*, 2008) suggests that a deficit of the central neural component contributes to the attenuation of arterial baroreflex in OVE26 type 1 diabetic mice. This is because they found that stimulation of the aortic depressor nerve induced a lesser magnitude of bradycardia in OVE26 type 1 diabetic mice as compared to sham mice, but the bradycardic response to vagal efferent stimulation was enhanced (Gu *et al.*, 2008). Immunoreactive study has shown that reduced c-Fos expression (an indicator of early cellular response to many extracellular signals) in the nucleus tractus solitarii links to the attenuated arterial baroreflex sensitivity in STZ-induced diabetic rats (Gouty *et al.*, 2001). In addition, Chen, et al (Chen *et al.*, 2008) have reported that neural firing activity of the nucleus tractus solitarii in STZ-induced diabetes is reduced, which is involved in the impaired arterial baroreflex function in STZ-induced diabetic rats. Furthermore, a chronic intracerebroventricular infusion of leptin (a hormone produced by fat cells and improving glucose utilization, Minokoshi *et al.*, 1999; Wang *et al.*, 1999) totally normalizes the impaired arterial baroreflex sensitivity in STZ-induced diabetic

rats (do Carmo *et al*., 2008), which indirectly suggests that impairment of the central neural system is associated with the arterial baroreflex dysfunction in type 1 diabetes. These findings allow us to assume the involvement of the impaired central neural integration in the blunted arterial baroreflex in type 1 diabetes even though there is no report focusing on the mechanisms responsible for the impairment of the central neural component of the arterial baroreflex.

3.3 Participation of autonomic neuroeffector component in the blunted arterial baroreflex in type 1 diabetes

The autonomic neuroeffector component of the arterial baroreflex includes intracardiac ganglia, parasympathetic efferents, and sympathetic efferents. Morphological studies have shown that there is a remarkable structural remodeling of the intracardiac ganglia (such as cellular contraction, cytoplasmic condensation, degenerated axons, reduced cell size and number) in STZ-induced diabetic rats (Kamal *et al*., 1991; Lund *et al*., 1992), mice (Lin *et al*., 2010), and diabetic patients (Tsujimura *et al*., 1986). Biochemical studies also found a decrease in acetylcholine (a neurotransmitter in both the central and parasympathetic nervous system) concentration in alloxan-induced diabetic rats (Kuntscherova & Vlk, 1970) and a reduced choline acetyltransferase activity (an enzyme producing acetylcholine) in the hearts of the STZ-induced diabetic rats (Lund *et al*., 1992). In addition, the function of the parasympathetic (vagal) efferent is reduced in STZ-induced diabetic rats (Maeda *et al*., 1995; Yagihashi, 1995). However, functional studies have reported normal, reduced, or enhanced heart rate response to vagal efferent nerve stimulation in diabetic animal models (Dall'Ago *et al*., 2007; de *et al*., 2002; Lin *et al*., 2010; Maeda *et al*., 1995; McDowell *et al*., 1994a). This discrepancy might be due to different animal species, experimental diabetic animal models, and time course of development of diabetes. Therefore, further studies are needed to explore whether the altered efferent component of the arterial baroreflex is responsible for the arterial baroreflex dysfunction in type 1 diabetes besides the arterial baroreceptor and central integration.

4. Conclusion

As a homeostatic mechanism, the arterial baroreflex normally alters heart rate and blood pressure in response to changes in arterial wall tension detected by the baroreceptors in the carotid sinus and aortic arch. As illustrated by the above evidence, arterial baroreflex impairment, a characteristic of the autonomic cardiovascular dysfunction is a frequent complication in type 1 diabetic patients and animal models. The arterial baroreflex dysfunction not only is an independent predictor for mortality of the type 1 diabetic patients, but also is associated with a poor prognosis and bad quality of life in the type 1 diabetic patients.

Although the mechanisms responsible for attenuated arterial baroreflex function in the type 1 diabetes are not yet fully understood, any part of the arterial baroreflex arc including an afferent limb, a central neural component, and an autonomic neuroeffector component can contribute to the arterial baroreflex dysfunction in the type 1 diabetic state. Especially at the level of the afferent limb, recent studies have revealed that aortic depressor nerve discharge and excitability of aortic baroreceptor neurons are blunted in the type 1 diabetic animals. HCN channels are significantly suppressed in the aortic baroreceptor neurons and are involved in the blunted baroreceptor neuron excitability in the type 1 diabetes. Angiotensin

II/AT_1 receptor-NADPH oxidase-superoxide signaling regulates this alteration of the HCN channels in the aortic baroreceptor neurons and consequently decreases the arterial baroreflex function. In addition, we also consider that angiotensin II/AT_1 receptor-NADPH oxidase-superoxide signaling affects the changes in the central neural and autonomic neuroeffector components beyond the afferent limb of the arterial baroreflex arc. These studies provide new information on the mechanisms underlying the impaired arterial baroreflex in the type 1 diabetes and unveil important pharmacological and genomic targets for improving the arterial baroreflex function and reducing the mortality in the type 1 diabetes.

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9

Microvascular and Macrovascular Complications in Children and Adolescents with Type 1 Diabetes

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1. Introduction

Diabetes mellitus is a serious chronic disorder of childhood and represents a major public health problem. Type 1 diabetes is the predominant form of diabetes during childhood and adolescence, accounting for about 90% of cases, although the growing epidemic of obesity has been associated with an increasing number of cases of childhood-onset type 2 diabetes (Craig et al., 2009; Patterson et al., 2009).

The global incidence of type 1 diabetes is increasing worldwide, at an annual rate of 3-5%, particularly in children under the age of 5 years, and this trend leads to a significant health burden (Patterson et al., 2009). Recent studies have shown that in European countries childhood-onset type 1 diabetes is associated with three to four fold increased mortality when compared with the general population (Asao et al., 2003; Skrivarhaug et al., 2006). Similar data emerged from a long-term study of a young cohort with type 1 diabetes in the USA, where mortality was 7 times higher than in the non-diabetic population (Secrest et al., 2010a). The high mortality reported for individuals with type 1 diabetes is mainly due to diabetes-related acute and chronic complications. As recently emerged from a large population-based cohort with long-standing childhood-onset type 1 diabetes, during the first decade of diabetes acute complications, such as diabetic ketoacidosis and hypoglycemia, are the main causes of death, being responsible for about 73% of cases, whereas during subsequent decades cardiovascular (CVD) and renal diseases become the main determinants of mortality (Secrest et al., 2010b).

Diabetes vascular complications are divided in micro- and macrovascular disease. Microvascular complications include nephropathy (DN), retinopathy (DR) and neuropathy, whereas macrovascualar complications refer to cardiovascular, cerebrovascular and peripheral vascular disease (Marshall and Flyvbjerg, 2006). As a result of vascular complications, diabetes is the leading cause of blindness in working age people, is responsible for up to 40% cases of renal failure and is a major determinant of cardiovascular morbidity and mortality (Marshall and Flyvbjerg, 2006).

2. Microvascular complications of type 1 diabetes

Diabetic microvascular complications result from damage to the microvasculature of the kidney, retina and neurons and they generally progress throughout different stages.

2.1 Diabetic nephropathy

2.1.1 Classification of diabetic nephropathy and structural kidney changes

The changing occurring in the kidney in patients with type 1 diabetes are generally classified in five stages (Mogensen, 1999). The first stage is characterized by increases in glomerular filtration rate (GFR) and kidney hypertrophy. During the second phase subtle morphological changes occur together with progressive increases in urinary albumin excretion within the normal range. The third stage, also called incipient nephropathy, is characterized by the development of microalbuminuria, defined as an albumin excretion rate (AER) between 30-300 mg/24h or 20-200 μg/min, and by more profound structural changes. During the fourth phase there is a further increase in AER leading to macroalbuminuria (AER >200 μg/min or >300 mg/24h) and a consistent fall in GFR. Without any treatment this phase leads to the final stage of end stage renal disease (ESRD).

Typical morphological changes occurring in the diabetic kidney are represented by diffuse glomerular basement membrane thickening, mesangial expansion, hyalinosis of the mesangium and arteriolar walls, broadening and effacement of podocyte foot processes, reduction in podocyte number, glomerulosclerosis and tubule-interstitial fibrosis (Osterby, 1992). These morphological changes develop years before the clinical appearance of DN and this is an alarming aspect, given that when the disease is clinically evident some of the structural damage is already irreversible.

Thickening of the basement membrane is a common bioptic finding related to DN and is associated with loss of glycosaminoglycans and associated negative charges, with consequent increased loss of anionic albumin (Fioretto and Mauer, 2007; Fioretto et al., 1994; Mauer and Najafian, 2006). A subsequent increase in the size of membrane pores leads to the development of non-selective proteinuria. An imbalance between the production and the degradation of mesangial matrix proteins, together with an increase in mesangial cells number, is responsible for mesangial expansion in DN (Fioretto and Mauer, 2007; Mauer and Najafian, 2006).

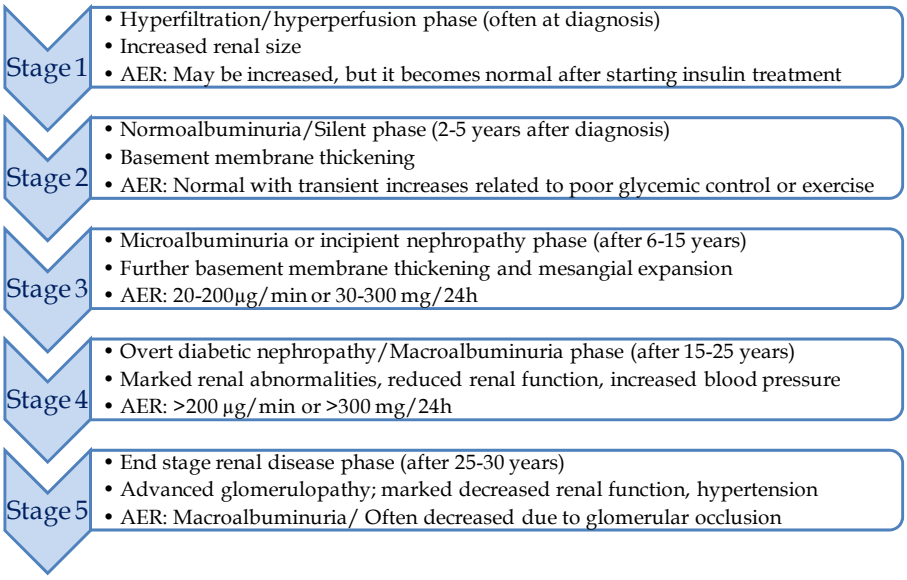

Fig. 1. Stages of diabetic nephropathy

Changes in the podocyte is another key feature of DN (Mauer and Najafian, 2006). Podocytes are highly specialised epithelial cells, interconnected by foot processes, which delimit the slit diaphragm, the main size-selective barrier in the glomerulus. The first detectable alteration in podocytes in the context of DN is a broadening and effacement of their foot processes (Kriz et al., 1998), which leads to a decrease in their density and number and a detachment from the glomerular basement membrane. The last phenomena are directly correlated to levels of albumin-creatinine ratio (ACR) and to the decline in GFR (Fioretto and Mauer, 2007). Podocytes can also undergo hypertrophy, apoptosis and increased synthesis of collagen IV, whereas there is a decreased synthesis of proteins, such as nephrin (Fioretto and Mauer, 2007).
Another characteristic of DN is the hyalinosis of the afferent and efferent arterioles in the glomerulus, due to the accumulation of complement components, fibrinogen, immunoglobulins, albumin and other plasma proteins (Fioretto and Mauer, 2007). Hypertrophy and sclerosis of the iuxtaglomerular apparatus is another frequent finding in DN (Mauer and Najafian, 2006).

2.1.2 Epidemiology of diabetic nepropathy

DN affects about one third of people with type 1 diabetes after a disease duration of 20 years and represents an important determinant of mortality (Jones et al., 2005). This has been recently confirmed in patients with both childhood-onset and adult-onset type 1 diabetes (Groop et al., 2009; Orchard et al., 2010). In a large cohort of adults with type 1 diabetes the presence of microalbuminuria, macroalbuminuria and ESRD was associated with 2.8, 9.2, and 18.3 times higher standardized mortality ratio, respectively (Groop et al., 2009). Similarly, Orchard *et al*. reported a standardized mortality ratio of 6.4, 12.5 and 29.8 for individuals with microalbuminuria, proteinuria and ESRD, respectively, in a cohort with childhood-onset type 1 diabetes followed longitudinally for 30 years (Orchard et al., 2010).
The natural history of DN has changed over the last decades and, whereas earlier landmark studies indicated that patients with microalbuminuria had a 60-85% risk of progressing to overt proteinuria within 6-14 years (Mogensen and Christensen, 1984; Parving et al., 1982; Viberti et al., 1982), more recent studies have reported a rate of progression of 30% over 10 years, and an increasing number of cases of regression to normoalbuminuria (31 to 58%) (de Boer et al., 2011; Hovind et al., 2004; Perkins et al., 2003). Regression to normoalbuminuria has been associated with a better metabolic control, a better lipid and blood pressure profile as well as with non-modifiable risk factors, such as younger age and shorter duration of microalbuminuria (Hovind et al., 2004; Perkins et al., 2003). These data are encouraging and highlight the positive effect of improvements in treatment, particularly glycemic (1993) and blood pressure control (Lewis et al., 1993), in influencing the natural history of DN. However, this changing trend may be also related to an overestimation of the rate of progression in earlier studies.
With regards to young people with childhood onset type 1 diabetes, recent studies from Sweden (Nordwall et al., 2004) and Australia (Mohsin et al., 2005) have shown a decreasing trend in DN. However, these positive results have not been consistently reported. In fact, data from Iceland (Tryggvason et al., 2005) and the UK (Amin et al., 2009) indicated an unchanged trend in the incidence of microalbuminuria and DN over the last decades.

2.1.3 Early manifestations of diabetic nephropathy in youth with type 1 diabetes

Microalbuminuria is the most common abnormal finding in children and adolescents with type 1 diabetes, whereas overt proteinuria is found in less than 1–1.5% of them (Jones et al., 1998; Schultz et al., 1999b). Microalbuminuria often develops during puberty, whereas its prevalence during prepubertal years is rare (Janner et al., 1994; Lawson et al., 1996; Norgaard et al., 1989; Rudberg et al., 1993). The overall prevalence of microalbuminuria in youth with type 1 diabetes ranges between 4 and 26% (Bojestig et al., 1996; Cook and Daneman, 1990; Dahlquist and Rudberg, 1987; Joner et al., 1992; Jones et al., 1998; Mathiesen et al., 1986; Moore and Shield, 2000; Norgaard et al., 1989; Olsen et al., 2000; Rudberg et al., 1993; Schultz et al., 1999b). This large variation in the prevalence of microalbuminuria is due to differences in study design, duration of diabetes, age range, and glycemic control. Most studies investigating microalbuminuria have been cross-sectional and clinic based, with only a few being longitudinal, but mainly with a retrospective design. Jones *et al.* (Jones et al., 1998) reported a prevalence of 14.5% during 8.5 years of diabetes duration, whereas Rudberg *et al.* (Rudberg et al., 1993) reported a prevalence of 24% after 15 years diabetes duration. The most recent data from the Oxford Regional Prospective Study (ORPS), a population-based inception cohort of children with type 1 diabetes, has shown a cumulative prevalence of microalbuminuria of 25.7% after 10 years and of 50.7% after 19 years of diabetes duration (Amin et al., 2008).

After puberty, rates of rise in albumin excretion tend to decline and, longitudinal studies suggest that microalbuminuria is persistent in only 50% of adolescents; whereas in the other 40-50% urinary albumin excretion returns into the normal range after 3-10 years from the onset of microalbuminuria (Amin et al., 2008; Gorman et al., 1999). However, with longer follow-up cases of transient or intermittent microalbuminuria may become persistent. In addition, although urinary albumin excretion may return into the normal range, the renal morphological changes associated with microalbuminuria can persist and increase the risk of its recurrence and progression (Steinke and Mauer, 2008). Few data are available on the rate of progression of microalbuminuria to macroalbuminuria in young people with type 1 diabetes. The ORPS has shown a progression rate of around 13% after 3.2 years from the onset of microalbuminuria, a rate similar to that reported in adults (Amin et al., 2008).

Another renal abnormality often detected in youth with type 1 diabetes is increased GFR (Amin et al., 2005; Rudberg et al., 1992). Hyperfiltration, which has been associated with increased renal size often precedes the onset of microalbuminuria (Amin et al., 2005; Rudberg et al., 1992). In some, although not all studies, increased GFR has emerged as an independent predictor of microalbuminuria, and a recent meta-analysis has reported a 2.7 increased risk of developing microalbuminuria associated with hyperfiltration (Magee et al., 2009).

2.2 Diabetic retinopathy

2.2.1 Classification and structural changes

DR begins with the appearance of non-proliferative retinal abnormalities, which then progress to sight-threatening proliferative lesions (Aiello et al., 1998; Williams and Pickup, 2004) . The early stage of DR is characterised by the development of capillary microaneuryms, which consist in small blind outgrowths of retinal capillaries developing in areas where the wall is weakened (Aiello et al., 1998; D'Amico, 1994; Williams and Pickup, 2004). As the retinal damage progresses, there is the appearance of non-proliferative abnormalities, including

hemorrhages, exudates and the development of vascular obstruction, intraretinal microvascular abnormalities, and infarction of the retinal nerve fibers causing cotton wool spots (Aiello et al., 1998; D'Amico, 1994; Williams and Pickup, 2004). Although this stage is not sight-threatening, it is highly predictive of progression to more advanced stages of retinopathy. Proliferative retinopathy is characterized by the development of new vessels, secondary to ischemia, on the surface of the retina and/or the optic disc (Aiello et al., 1998; D'Amico, 1994; Williams and Pickup, 2004). These new vessels can bleed into the vitroretinal space, and cause visual loss. In addition, the subsequent formation of fibrous tissue can cause tractional retinal detachment (Williams and Pickup, 2004). This stage is associated with a high risk for visual impairment related to hemorrhages and retinal detachment.
Diabetic macular oedema can complicate both non-proliferative and proliferative retinopathy and is a serious cause of vision loss in patients with diabetes (Ciulla et al., 2003). It is characterized by increased microvascular permeability and deposition of hard retinal exudates (Ciulla et al., 2003). This stage involves the breakdown of the blood-retinal barrier, with leakage of plasma from small blood vessels in the macula. Swelling of the macula is then followed by deposition of hard retinal exudates as a consequence of deposition of lipids and lipoproteins following plasma re-absorption (Ciulla et al., 2003).

Proposed disease severity level	Dilated ophthalmoscopy findings
No apparent retinopathy	No abnormalities
Mild nonproliferative DR	Microaneurysms only
Moderate nonproliferative DR	More than just microaneurysms, but less than severe nonproliferative DR
Severe nonproliferative DR	No signs of proliferative DR, with any of the following: - More than 20 intraretinal hemorrhages in each of four quadrants - Definite venous beading in two or more quadrants - Prominent intaretinal microvascular anomalies in one or more quadrants
Proliferative DR	One or more of the following: - Neovascularization - Vitreous or preretinal hemorrhage

Table 1. International clinical DR disease severity scale

Retinal damage in diabetes is mainly due to leakage of retinal blood vessels and inadequate retinal perfusion (Aiello et al., 1998; D'Amico, 1994; Williams and Pickup, 2004). It has been suggested that one of the initial alterations in the retinal hemodynamic is represented by retinal vasodilatation and hyperperfusion, due to hypoxia and increased release of nitric oxide (Joussen et al., 2004). This is followed by an impairment of the retinal vascular autoregulation with increased tension in the epithelial wall and increased vascular permeability, which, in turn, cause vascular leakage and aneurysm formation (Kohner et al., 1995; Scherrer et al., 1994).

Early structural abnormalities in the retinal microvasculature are characterised by thickening of the basement membrane, loss of endothelial cells and pericytes and increased capillary permeability (Kohner et al., 1995). An important characteristic of retinopathy is the loss of pericytes from the retinal capillaries (D'Amico, 1994; Feng et al., 2007; Frank, 2004). Pericytes are contractile cells which have an important role in the regulation of the capillary blood flow and their loss has been associated with the development of retinal microaneurysms (Feng et al., 2007; Kuwabara and Cogan, 1963). The loss of pericytes, associated with that of capillary endothelial cells, contributes to the disruption of the integrity of the blood–retinal barrier. Thickening of the capillary basement membrane and deposition of extracellular matrix components are additional mechanisms contributing to the alterations in the retinal blood flow (Feng et al., 2007).
Retinal leukostasis has been also associated with DR and, in particular, with capillary occlusion and the consequent appearance of retinal areas of non perfusion (Aiello et al., 1998; D'Amico, 1994; Williams and Pickup, 2004). These alterations contribute, in turn, to retinal ischemia, which is a potent stimulus for vascular neoformation (Miyamoto and Ogura, 1999).

2.2.2 Epidemiology of retinopathy

DR is the leading cause of blindness in people of working age in Western countries (Ciulla et al., 2003). The prevalence of DR increases with age and its was approximately 17.7 per 100 people with diabetes in the year 2005 (Deshpande et al., 2008). DR can be diagnosed already after 5 years from the onset of diabetes, and almost all patients will show variable degrees of DR after 20 years diabetes duration.
Recent data from the Wisconsin Epidemiologic Study of DR have shown that the 25-year cumulative incidence of visual impairment in adults with type 1 diabetes was 13% and that of severe visual impairment was 3%. Patients with onset of diabetes during more recent years have a lower prevalence of visual impairment when compared with those diagnosed in the past, independently of duration of diabetes (Klein et al., 2010). This is in agreement with some other recent studies reporting a declining incidence of retinopathy and other microvascular complications (Nathan et al., 2009; Nordwall et al., 2004).

2.2.3 Early manifestations of retinopathy in youth with type 1 diabetes

Early stages of DR can be detected in young people with type 1 diabetes, as shown by a population based study from Australia, where early background retinopathy was detected in 24% of the study population after 6-year diabetes duration (Donaghue et al., 2005). Similarly, in a Swedish study retinopathy was detected in 27% of young patients after 13 years of duration (Nordwall et al., 2006). Children with type 1 diabetes under the age of 10 years are at minimal risk of DR, but the prevalence rate increases after 5 years from diagnosis in post-pubertal patients (Klein et al., 1984). In an incident cohort, early retinopathy was detected in 12% of prepubertal children compared to 29% of adolescents, after 6 year type 1 diabetes duration (Donaghue et al., 2005). Interestingly, adolescents with type 1 diabetes have a higher risk of progression to sight-threatening DR when compared to adults and the progression may be particularly rapid when glycemic control is poor (Maguire et al., 2005). As for DN, cases of regression have also been reported for DR (Maguire et al., 2005).

2.3 Diabetic neuropathy

Diabetic neuropathy is the most common neuropathy in industrialized countries, and it is associated with a wide range of clinical manifestations (Tesfaye et al., 2010). Diabetic neuropathy is defined as a clinical or subclinical disorder, without any additional causes other than diabetes, and can be either somatic or autonomic (Boulton et al., 2005). Early diagnosis and treatment of diabetic neuropathy are important given that peripheral neuropathy is associated with a high risk of feet injury in patients with type 1 diabetes (Boulton et al., 2005). In addition, diabetic autonomic neuropathy can lead to a significantly increased morbidity and mortality, mainly when involves the cardiovascular system (Boulton et al., 2005).

2.3.1 Classification and epidemiological data of diabetic neuropathy

Chronic distal symmetric polyneuropathy (DPN) is the most common form of diabetic neuropathy and affects 30-50% of patients with type 1 diabetes and can be asymptomatic in up to 50% of them (Tesfaye et al., 2010). DPN implies symmetric damage of peripheral small sensory and large motor nerve fibres. It generally starts from the most distal end of the feet and then extend proximally over time and can lead to foot ulceration and amputation of lower limbs (Boulton et al., 2005). Dysfunction of peripheral small nerve fibres is characterised by parasthesiae, burning, and deep aching pain. If larger nerve fibres are affected, vibration, light touch and joint position senses are impaired, and tendon reflexes are absent (Boulton et al., 2005). Less common forms of diabetic somatic neuropathy include focal or multifocal neuropathies, which are characterized by entrapment of a peripheral nerve commonly the median, ulnar or peroneal nerve (Boulton et al., 2005).

Diabetic autonomic neuropathy is a disorder of the autonomic nervous system in the context of diabetes and can affect the cardiovascular, gastrointestinal, urogenital systems and the sudomotor function (Tesfaye et al., 2010). Autonomic neuropathy can be observed in around 20% of asymptomatic patients with diabetes (Tesfaye et al., 2010). Clinical symptoms of autonomic neuropathy do not generally occur until long after the onset of diabetes. Subclinical autonomic dysfunction can, however, occur within two years of diagnosis in patients with type 1 diabetes (Boulton et al., 2005). Dysautonomic features may reflect the involvement of different systems and manifest as postural hypotension and orthostatic lightheadedness, gastroparesis, gastric fullness, early satiety, sexual dysfunction, bladder dysfunction, gustatory sweating or anhidrosis and pupillomotor dysfunction (Boulton et al., 2005).

Peripheral neuropathies *Generalized symmetric polyneuropathies* - Acute sensory - Chronic sensorimotor - Autonomic *Focal and multifocal neuropathies* - Cranial - Truncal - Focal limb - Proximal motor	**Autonomic neuropathy** - *Cardiovascular:* Postural hypotension, resting tachicardia, exercise intolerance - *Genitourinary:* Bladder dysfunction, Erectile dysfunction - *Gastrointestinal:* Gastric paresis, constipation/diarrhea, fecal inconinence - *Sudomotor:* Gustatory sweating, Anhidrosis, Heat intolerance, Dry skin - *Metabolic:* hypoglycemia unawareness - *Pupillomotor dysfunction*

Table 2. Neuropathies in diabetes

Diabetic neuropathy is characterized by a reduction in the number of fibers, degeneration of the myelin sheath as well as changes affecting the endoneurial connective tissue, vessels and perineurium (Greene et al., 1992). The process of nerve demyelination may progress to Wallerian degeneration, in which the nerve axon is also injured and the distal part of the axon dies (Greene et al., 1992).

2.3.2 Early signs of diabetic neuropathy in youth with type 1 diabetes

In the most comprehensive epidemiological studies involving both adult and pediatric patients, DPN was detected in 9 to 58% of the study populations (Boulton et al., 2005). Data on autonomic neuropathy indicate a prevalence ranging from 14% to 75%, with a high number of youth with type 1 diabetes presenting suclinical signs of autonomic dysfunction, even after a short duration of T1D. This variability across different studies is mainly related to differences in the characteristics of the study cohorts as well as to the use of different testing modalities and different criteria and cut off values (Trotta et al., 2004; Verrotti et al., 2009).

3. Macrovascular complications of type 1 diabetes

Patients with type 1 diabetes have an increased risk of developing cardiovascular disease (CVD) relative to the nondiabetic population, and premature atherosclerosis represents the main cause of morbidity and mortality in type 1 diabetes populations (Laing et al., 2003).
There is extensive evidence in support of the concept that atherosclerosis begins early in life (Ross, 1993) and therefore identification of CVD risk factors and preventive strategies should be started during childhood and adolescence (Dahl-Jorgensen et al., 2005). Children and adolescents with type 1 diabetes represent a high risk population with regards to CVD, given that cardiovascular risk factors are common among them (Dahl-Jorgensen et al., 2005; Margeirsdottir et al., 2008; van Vliet et al.) and they can contribute to their poor long-term prognosis (Skrivarhaug et al., 2006). A recent study has shown that as many as 86% of youth with type 1 diabetes has at least one, 45% at least two and 15% at least three CVD risk factors, including high HbA1c, high blood pressure, dyslipidemia, smoking and family history of CVD events (Margeirsdottir et al., 2008). These data are alarming given that it is well known that CVD risk factors can persist or track over time (Berenson, 2002) and therefore contribute to the overall burden associated with type 1 diabetes.

3.1 Early signs of macrovascular disease in youth with type 1 diabetes

The early stages of the atherosclerotic process are silent, but autopsy studies have detected early structural alterations in the arteries of youth, where they have been associated with the same risk factors than in adults (Berenson et al., 1998). The earliest recognizable pathologic intimal lesions are the fatty streaks, which make their appearance in the aorta of children even before 3 years of age (McGill et al., 2000; Williams et al., 2002). Fatty streaks represent an early manifestation of lipid accumulation in the vessel wall and they have been associated with increased cholesterol levels as well as with hyperglycemia (McGill et al., 2000).
Technology progresses over the last years have made possible to look for early surrogate markers of atherosclerotic vascular disease. These markers are represented by structural alterations such as increased intima-media thickness as well as functional changes represented by decreased flow-mediated dilatation and increased arterial stiffness, as detected by pulse wave velocity (Dahl-Jorgensen et al., 2005).

Several studies have consistently shown that children and adolescents with type 1 diabetes present signs of endothelial dysfunction, as measured by flow-mediated dilation in the brachial artery (Jarvisalo et al., 2004; Singh et al., 2003). In addition, increased aortic and carotid intima-media thickness has been reported in children with type 1 diabetes (Harrington et al., 2010; Jarvisalo et al., 2001). Besides, markers of inflammation and oxidative stress are significantly increased in youth with type 1 diabetes when compared with age-matched controls and they can mediate vascular damage (Snell-Bergeon et al., 2010).
Progression of CVD in youth with type 1 diabetes can be more aggressive than in adults with diabetes, therefore highlighting the importance of early preventive strategies (Dahl-Jorgensen et al., 2005).

Early manifestations of CVD in youth with type 1 diabetes
• Increased inflammatory markers
• Increased oxidative stress
• Increased intima-media thickness
• Decreased flow-mediated dilatation
• Increased pulse wave velocity

Table 3. Early manifestations of CVD in youth with type 1 diabetes

4. Pathogenesis of micro- and macrovascular complications

Vascular complications in the context of diabetes are the result of an interplay between hemodynamic and metabolic factors and the consequent activation of common intermediate pathways, associated with increased synthesis and release of growth factors, cytokines, chemokines and oxidant species, which are all final mediators of vascular damage (Cooper, 2001).
A strong association exists between the presence of micro- and macrovascular complications in people with type 1 diabetes (Girach and Vignati, 2006). In particular, it is well known that DN is a key risk factor for cardiovascular complications and many patients with renal impairment die of CVD-related causes even before developing ESRD (Groop et al., 2009). In addition, there is growing evidence suggesting that increases in albumin excretion, even within the normal range, in the general population as well as in people with type 1 diabetes, represents a determinant of CVD (Klausen et al., 2004). The link between micro- and macrovascular disease could be represented by endothelial dysfunction, as an underlining feature of both processes, as well as by a certain degree of inflammation, which has been associated to the presence of both micro- and macrovascular complications (Schalkwijk and Stehouwer, 2005).

4.1 Glycemic control

Chronic hyperglycemia is known to activate several deleterious pathways implicated in the damage of vessels: protein glycation, increased glucose flux through alternative polyol and hexosamine pathways, increased oxidative stress, which then stimulate secondary intracellular signaling pathways leading to production of growth factors, cytokines and inflammatory factors (Brownlee, 2001).
Several epidemiological studies have shown a direct and strong association between long-term glycemic control, as expressed by HbA1c levels, and the risk of developing nephropathy, retinopathy and neuropathy (Amin et al., 2008; Danne et al., 1998; Gallego et al., 2008; Nordwall et al., 2009). In addition, HbA1c has been shown to be a key determinant also of early signs of atherosclerosis (McGill et al., 1995).

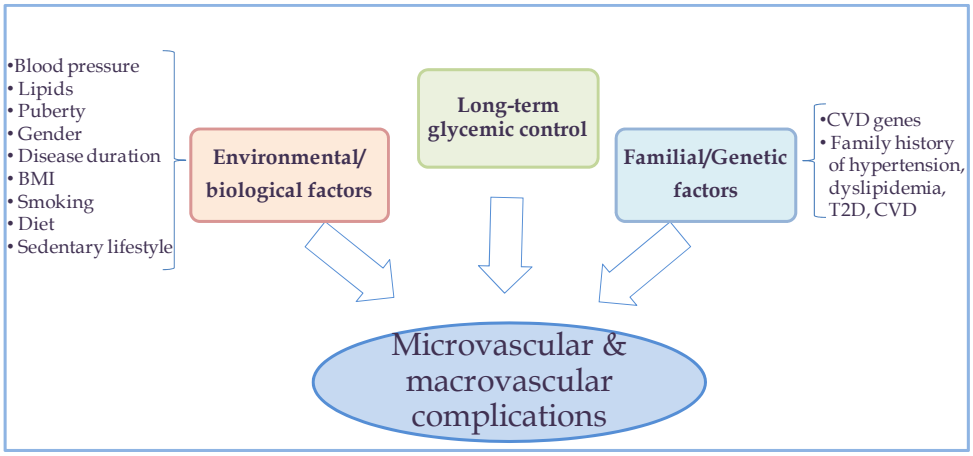

Fig. 2. Risk factors for the development of vascular complications in adolescents with type 1 diabetes

The Diabetes Control and Complications Trial (DCCT) and the Epidemiology of Diabetes Interventions and Complications (EDIC) studies have clearly shown the beneficial effect of strict glycemic control in reducing the risk of microvascular and macrovascular complications in subjects with type 1 diabetes (1993; 2003). In the adolescent cohort of the DCCT, a positive effect of improved glycemic control on complication risk was obtained. Intensive insulin therapy reduced the risk for the development and progression of DR by 76% and 54%; the occurrence of microalbuminuria and proteinuria by 39% and 54% and that of clinical neuropathy by 60% when compared to the conventional treated group (1994). Data from the DCCT/EDIC study also showed that intensive insulin therapy reduced the risk for any cardiovascular disease by 42% and of nonfatal myocardial infarction, stroke or death from CVD cause by 57% (Nathan et al., 2005). The beneficial effect of intensive insulin treatment in patients with childhood-onset type 1 diabetes also emerged from the Oslo study, where long-term glycemic control predicted coronary atherosclerosis (Larsen et al., 2002).

In addition, the EDIC study raised the important concept of 'metabolic memory'; in other words although after the end of the DCCT HbA1c levels became comparable between the intensively and conventionally treated groups, patients belonging to the first group still kept an advantage from prior better HbA1c levels (2003). Therefore, the EDIC data highlighted the need of implementing intensive management as soon as type 1 diabetes is diagnosed. This was further supported by recent data related to DR, showing that, although at year 10 of the EDIC there was still a decrease in DR progression risk in the intensively treated adult group, in the adolescent cohort retinopathy progression at year 10 of the EDIC did not differ between the previous DCCT intensively and conventionally treated groups, thus indicating loss of the metabolic memory (White et al., 2010). Interestingly, 79% of the difference in the metabolic effect between adults and adolescents after 10 years from the end of the DCCT was due to the difference in mean HbA1c levels during the DCCT between the two cohorts. This 1% difference, which did not seem to play a major role during the DCCT and early EDIC years with regards to the outcomes, seems to be a major player in the long run (White et al., 2010).

4.2 Blood pressure and plasma lipids

Elevated blood pressure and alterations in its circadian rhythm are common findings in people with type 1 diabetes and have been associated with the risk of developing vascular complications (Gallego et al., 2008; Marcovecchio et al., 2009b; Tesfaye et al., 1996). Increases in blood pressure have been found to precede or occur concomitant with the appearance of microalbuminuria in adolescents with type 1 diabetes (Marcovecchio et al., 2009b; Schultz et al., 2001). Similarly, higher than normal systolic and diastolic blood pressure independently contribute to the development of DR (Gallego et al., 2008). Blood pressure has been also associated with early markers of atherosclerosis (Dahl-Jorgensen et al., 2005).

Lipid abnormalities have also been linked to the development and progression of micro- and macrovascular complications in adolescents with diabetes (Dahl-Jorgensen et al., 2005; Kordonouri et al., 1996; Marcovecchio et al., 2009a).

4.3 Duration and age at onset of type 1 diabetes

Duration of diabetes is another major determinant of complication risk. Although vascular complications rarely appear before puberty, prepubertal duration of diabetes is an important determinant for their development (Coonrod et al., 1993; Orchard et al., 1990). Patients with type 1 diabetes from early childhood and especially those diagnosed under five years of age seem to have slightly delayed onset of persistent microalbuminuria during the first 10 to 15 years duration compared with patients diagnosed later in childhood or during puberty. However, this initial protective effect of a younger age at diagnosis disappears over time (Amin et al., 2008; Donaghue et al., 2003). After 15 years of diabetes duration the risk of developing microalbuminuria is similar between subjects diagnosed with diabetes before 5 years of age when compared with those diagnosed between 5-11 years of age or after the age of 11 years, suggesting that age at the onset of diabetes does not influence the overall risk for microalbuminuria (Amin et al., 2008). A recent study has highlighted that also for DR, although patients diagnosed at a young age have a longer time free of proliferative retinopathy, this advantage then gradually disappears and youth diagnosed before the age of 15 years have a higher risk of proliferative retinopathy when compared with those diagnosed when aged 15-40 years (Hietala et al., 2010).

4.4 Puberty

Puberty is an important factor implicated in the development and progression of vascular complications. Poor glycemic control is a common finding among adolescents with type 1 diabetes (1994; Holl et al., 2003). In addition, puberty is associated with a decrease in insulin sensitivity, and adolescents with type 1 diabetes are more insulin resistant when compared with healthy controls (Dunger, 1992). Rapid growth, hormonal and metabolic changes characterise this period of life and can influence complications risk (Dunger, 1992).

4.5 Gender

A gender dimorphism has been reported for vascular complications. In particular, during adolescence the risk for microalbuminuria is higher in female than in male subjects with comparable glycemic control (Amin et al., 2008; Jones et al., 1998), whereas among adults with type 1 diabetes the risk is higher for males (Hovind et al., 2004). These differences have been related to variations in the hormonal milieu and to a higher degree of insulin resistance in girls (Schultz et al., 1999a).

4.6 Other factors

Genetic factors represent another important contributing factor for the development of vascular complications, as suggested by their familial clustering, and by the observation that only a subset of patients with poor glycemic control develop severe long-term complications (1997). Family history of cardiovascular risk factors is associated with increased risk of microvascular complications in the offspring (Monti et al., 2007; Seaquist et al., 1989).

A high body mass index (BMI) represents another potential risk factor for microvascular complications in youth with type 1 diabetes (Stone et al., 2006). In addition, obesity is a well-known risk factor for cardiovascular disease (Dahl-Jorgensen et al., 2005).

Environmental factors, including diet and lifestyle, can also contribute to the risk of developing vascular complications. Smoking in people with type 1 diabetes has been associated with an increased risk of developing vascular complications (Chase et al., 1991; Mohamed et al., 2007).

5. Management of microvascular and macrovascular complications

5.1 Screening

Diabetic microvascular and macrovascular complications are often asymptomatic during their early stages, and once symptoms develop, they may be difficult to reverse. Therefore, longitudinal repeated screening for vascular complications initiated during early adolescence is currently recommended

The American Diabetes Association (ADA) recommends to start screening in patients with type 1 diabetes aged 10 years or older after a disease duration of 3-5 years, with yearly follow-up (Silverstein et al., 2005). The International Society for Pediatric and Adolescent Diabetes (ISPAD) recommends screening from the age of 11 years in those with 2 year diabetes duration and from 9 years in those with a 5 year duration for both nephropathy and retinopathy (Donaghue et al., 2007). Measurement of urinary albumin excretion is the basis for early detection of microalbuminuria and can be achieved with: 1) 24-hour urine collection; 2) overnight timed urine collections; 3) albumin-creatinine ratio (ACR) or albumin concentration on a early morning spot urinary sample (Donaghue et al., 2007). 24-hour or timed urine collections are often difficult to collect in children and adolescents. Assessing ACR in early morning urines is the easiest method to carry out in an office setting and it generally provides accurate information (Donaghue et al., 2007).

With regards to DR, several techniques can be used, including direct and indirect ophthalmoscopy, stereoscopic digital and color film based fundus photography, mydriatic or nonmydriatic digital color or monochromatic single-field photography. The best technique to identify and grade retinopathy is represented by retinal photography, through dilated pupils, but dilated indirect ophtalmoscopy associated with biomicroscopy is an acceptable alternative (Ciulla et al., 2003).

In contrast to well-established criteria for when starting screening for DN and DR, it is unclear when to commence screening for neuropathy. History and physical examination are generally the recommended methods of screening (Donaghue et al., 2007). Clinical examination, including history of pain, paresthesia, numbness and physical examination of ankle reflexes and vibration and light touch sensation, is a fundamental part of screening, although not being as sensitive or specific as nerve conduction studies (Donaghue et al., 2007). Autonomic neuropathy can be assessed with specific autonomic nerve tests, such as heart rate response to deep breathing, standing from a lying position, Valsalva maneuver, heart rate variations at

rest, QT interval, postural changes in BP and pupil responses to light and dark adaptation (Donaghue et al., 2007). These tests need to be carefully standardized and therefore they are largely used as screening methods for complications at a population level.
Additional screening concerns risk factors for CVD, such as dyslipidemia and hypertension. Blood lipids should be checked soon after diagnosis and if normal then repeated after 5 years (Donaghue et al., 2007). Office blood pressure should be assessed annually and in case of abnormal values, hypertension needs to be confirmed by ambulatory blood pressure monitoring.

When to start • Microvascular complications: at age 11 with 2 year duration of type 1 diabetes or from age 9 with 5 year duration (for nephropathy and retinopathy; unclear for neuropathy) • Macrovascular complications: after 12 years
Screening Method: *1) Diabetic nephropathy* • Annual albumin-creatinine ratio in a spot urine sample or first morning albumin concentration *2) Diabetic retinopathy* • Annual dilated fundus ophthalmoscopy or fundal photography *3) Diabetic neuropathy* • History and physical examination • Nerve conduction and autonomic tests *4) Macrovascular disease* • Annual assessments of blood pressure • Assessment of lipid levels every 5 years

Table 4. Screening for micro- and macrovascular complications in youth with type 1 diabetes

5.2 Interventions

Improving glycemic control is the cornerstone of treatment strategies aiming at reducing the development and progression of microvascular and macrovascular complications. The DCCT and EDIC study clearly showed the importance of a strict glycemic control both in adults and in adolescents with type 1 diabetes (1994). The importance of keeping HbA1c within targets has also been highlighted by other studies, but similarly to the DCCT, subsequent studies have confirmed the difficulties encountered when dealing with young people with type 1 diabetes (Holl et al., 2003; Petitti et al., 2009). Tight glycemic control in the DCCT and subsequent studies was associated with a higher risk of complications, such as hypoglycemia and weight gain (1994). In addition, poor compliance is an important issue to be taken into account in adolescents with type 1 diabetes. Furthermore, other factors besides insulin therapy are relevant for metabolic control, such as dietary habits, education, family interactions, cultural and psychological aspects (Holl et al., 2003), and therefore they need to be taken into account in order to define a successful treatment plan.
Treatment with angiotensin converting enzyme inhibitors (ACEIs) is recommended when hypertension is confirmed (Donaghue et al., 2007). ACEIs are treatment of choice in adults with microalbuminuria, based on evidence that their use decrease the rate of progression and even increase rates of regression of microalbuminuria (Lewis et al., 1993). A beneficial

effect of anti-hypertensive treatment, and in particular of treatment with ACEIs, has also been demonstrated for DR (Chaturvedi et al., 1998). The EURODIAB Controlled Trial of Lisinopril in type 1 diabetes showed a significant effect of lisinopril in reducing by around 50% the progression of retinopathy in normotensive and normo- or microalbuminuric patients (Chaturvedi et al., 1998). However, there is no universal recommendation for the use of ACEIs in children and adolescents with microalbuminuria. The ADA recommends to start treatment with ACEIs in presence of persistent microalbuminuria (Silverstein et al., 2005). Similarly, the recent ISPAD guidelines suggest to use ACEIs or angiotensin receptor blockers in presence of persistent microalbuminuria, in order to prevent progression to proteinuria (Donaghue et al., 2007), even though the lack of evidence in this context is acknowledged.

Dyslipidemia should be managed firstly with improvements in glycemic control and dietary changes and, in case of persistence of high cholesterol levels, treatment with statins should be considered, although there is no enough evidence of their use in children apart from those with familial hypercholesterolemia (Donaghue et al., 2007). However, in adults statins have been shown to be effective in the primary and secondary prevention of major cardiovascular events (2002).

Adolescents with type 1 diabetes need to be persuaded to avoid smoking. In addition, it is of paramount importance also to avoid increases in BMI, given that adiposity is a well known risk factor for cardiovascular complications, and in some studies it has also been associated with microvascular complications of type 1 diabetes (Donaghue et al., 2007).

Targets to reduce diabetic vascular complications
• HbA1c: ≤7.5% (without severe hypoglycemia)
• LDL cholesterol: <2.6mmol/l
• HDL cholesterol: >1.1mmol/l
• Triglycerides: <1.7mmol/l
• Blood pressure: <90th percentile for age, sex and height
• BMI <95th centile
• Avoid smoking
• Physical activity: moderate: >1hr/day
• Sedentary activities: <2hr/day
• Healthy diet

Table 5. Recommendations to reduce diabetic vascular complications (ISPAD guideline 2006-2007)

New potential therapeutic possibilities for the treatment of vascular complications are emerging and they include drugs targeting specific pathways implicated in their pathogenesis. These include inhibitors of aldose reductase, inhibitors of protein kinase C, antagonists of advanced glycation end-products, glycosaminoglycans, inhibitors of growth factors and anti-oxidants (Soro-Paavonen and Forbes, 2006). Up to now, there are no definitive data to recommend the use of these new potential therapies but the overall objective of targeting specific metabolic and hemodynamic pathways implicated in the pathogenesis of diabetic vascular complications could lead to validation of these classes of drugs and discovery of novel pharmaceuticals.

6. Conclusions

The risk for micro- and macrovascular complications is high in young patients with childhood-onset type 1 diabetes and their development negatively influence their long-term prognosis.

Early identification of risk factors and prevention of diabetes complications are of paramount importance in children and adolescents. Diabetic vascular complications are often asymptomatic during their early stages, and once symptoms develop, it can be difficult to reverse them. Therefore, screening for vascular complications started during early adolescence, as currently recommended, is essential. Identification of risk factors and subclinical signs of complications is of paramount importance for the early implementation of preventive and therapeutic strategies, which could change the course of vascular complications and improve the prognosis of youth with diabetes. Efforts should be made for optimizing glycemic control, to keep blood pressure and lipid levels within target levels, to avoid smoking, promote exercise and a healthy diet. Future studies are required to test the efficacy and safety of new therapies, which could target specific metabolic or hemodynamic pathways implicated in the pathogenesis of diabetic complications.

Further advances in genomics, proteomics and in other 'omics' and the integration of the findings of these different sciences will hopefully allow a better understanding of the pathogenesis of diabetic vascular complications in the near future and will potentially lead to a personalized medicine for young patients with diabetes

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10

Type 1 Diabetes Mellitus: Redefining the Future of Cardiovascular Complications with Novel Treatments

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1. Introduction

Diabetes Mellitus (DM) is a disease that was identified centuries ago, in around 1500 BC (King and Rubin, 2003). The word 'Diabetes' means "running through" (Holt, 2004) which is used to describe the excessive output of urine in this disease. "Mellitus" meaning "sweet" identifies the nature of the urine in patients suffering from DM (Widmaier *et al*., 2004). Thomas Willis was the first to differentiate DM from other polyurias in 1674 (Eknoyan and Nagy, 2005). In 1776, Matthew Dobson demonstrated that the sugar present in the urine was also present in the blood and was associated with its rise (Holt, 2004). In 1848, Claude Bernard's experiments on the liver showed that glycogen stored in the liver produced sugar and he hypothesized that glycogenolysis caused the disease. Although his discovery that "sugar production is a normal function of the animal" was revolutionary, it did not quite explain the etiology of the disease. In 1889, Oskar Minkowski confirmed that the ablation of the pancreas in dogs resulted in DM (Farmer, 1952). Frederick Banting and Charles Best were the medical scientists that discovered insulin, later in 1921, the lack of which, it was made clear, caused DM (Voet and Voet, 2004). They used the extract of a dog's fresh pancreas and demonstrated that upon administration of 10mL of extract to blood, blood glucose level is decreased from 0.3% to 0.17% (Rosenfeld, 2002). Some 30 years later, in 1953, Frederick Sanger was able to determine the complete amino acid sequence of the protein for the first time in history (Boron and Boulpaep, 2009).

Today, DM is defined as a carbohydrate disorder characterized by impaired insulin secretion and/or peripheral insulin resistance leading to hyperglycemia (Beers and Berkow. 1999). It is considered to be the third leading cause of death after heart disease and cancer in the United States (Voet and Voet, 2004) and its incidence is expected to rise to 366 million people by the year 2030 (Wild *et al*., 2004).

Diabetic patients have symptoms such as, thirst, polyuria, blurring of vision and weight loss. In extreme cases, ketoacidosis may develop leading to coma and ultimately death (Alberti and Zimmet, 1998). Diabetes Mellitus is classified according to etiology to two major types:

1.1 Type 1 diabetes mellitus

Although both type 1 Diabetes Mellitus (type 1 DM) and type 2 Diabetes Mellitus (type 2 DM) are characterized clinically by hyperglycemia, they have their differences. Type 1 DM

occurs commonly during childhood or adolescence therefore also named juvenile onset DM and may develop diabetic ketoacidosis (Beers and Berkow, 1999). Of all diabetes cases, 10% are type 1 DM (Holt, 2004). It includes all autoimmune and idiopathic causes of insulin-secreting β cell destruction resulting in absolute insulin deficiency (Alberti and Zimmet, 1998). The patient is usually genetically predisposed to DM, however, an environmental insult, such as a virus is needed to trigger the pathological process of the disease (Wilson *et al.* 1991). It is evident that CD4+ and CD8+ T lymphocytes are activated in the pancreatic lymph node and later infiltrate the pancreas causing inflammation (insulitis) (Yang and Santamaria, 2003). During this time, the body develops an immune response that sees pancreatic islet cells as 'nonself' and starts destroying its β cells (Wilson *et al.,* 1991). Specific causes of β cell destruction, such as cystic fibrosis or mitochondrial defects, are excluded from this classification (Alberti and Zimmet, 1998). Recently, type 1 DM was subdivided into type 1 A and type 1 B. Type 1A diabetes mellitus is described as a disease with immune mediated, selective destruction of insulin producing β cells, with the presence of anti-islet autoantibodies. Whereas type 1B diabetes mellitus, exhibits inflammation of the pancreas, but lack of anti-islet autoantibodies (Rhoades and Bell, 2009). Patients with type 1 DM require daily subcutaneous insulin administration as a treatment (Seifter *et al.,* 2005). Administration of exogenous insulin cannot be compared to the fine control of minute to minute insulin secretion that the pancreas provides (Hakim, 2002); for that reason, human islet cell transplantation has been accepted and applied as an alternative treatment for patients with type 1 DM (George, 2009).

1.2 Type 2 diabetes mellitus

Type 2 DM is also known "Adult-onset Diabetes" (Beers *et al.,* 2006), and represents 90-95% of diabetic patients (Creager *et al.*, 2003; Seifter *et al.*, 2005). This percentage is expected to increase in the year of 2025 to reach 300 million diabetic patients (Rhoades and Bell, 2009) due to sedentary lifestyle and increase in obesity in addition to age progression (Boron and Boulpaep, 2009; King *et al.,* 2005). As the intake of glucose increases insulin secretion is elevated. After a while, insulin secretion becomes inadequate due to peripheral resistance of insulin receptors (Beers *et al.*, 2006; King *et al.,* 2005). Insulin resistance decreases glucose-mediated insulin transport and metabolism (Rhoades and Bell, 2009), resulting in a defect of the compensatory insulin secretion (Mcphee *et al.*, 2008). It is worth noting that in type 2 DM reactive oxygen species are generated resulting in endothelial dysfunction, cardiovascular complications and renal disease (Hayashi *et al.*, 2010; Seifter *et al.*, 2005).
Insulin resistance develops when insulin signaling pathway is interfered in adipose, skeletal and hepatic cells (Seifter *et al.,* 2005). As a result of insulin resistance, glucose transport inside adipocytes and skeletal muscle is reduced and the suppression of glucose output from the liver is impaired. (Rhoades and Bell, 2009)

2. Complications of diabetes mellitus

Chronic hyperglycemia causes blood vessels injury (Seifter *et al.*, 2005) which is divided into two types depending on the size of vessels injured. Small vessels injury in diabetes is a microvascular complication whereas injury of large blood vessels determines a macrovascular complication (Hayashi *et al.*, 2010, Kalani, 2008). Microvascular and macrovascular complications occur in DM both types 1 and 2 (Retenakaran and Zinman,

2008; Rhoades and Bell, 2009). These injuries cause on the long run acceleration in atherosclerotic formation (Seifter *et al*., 2005). In type 1 diabetic patients, hyperglycemia has been correlated with a variety of events in cardiovascular pathology, initiated with endothelial dysfunction and progressed to develop arterial stiffness (Tabit *et al*., 2010). Thus, a relation has been clearly established to link glycemia with cardiovascular events in type 1 DM, based on the fact that glycemia itself is the only factor mediating a risk factor for cardiovascular risk in the absence of other risk factors (Retenakaran and Zinman, 2008). One way of reducing cardiovascular risk and renal outcomes in type 1 DM is to initiate an early intensive therapy for its management.

2.1 Microvascular complications

Microvascular complications include nephropathy, retinopathy and neuropathy (Coccheri, 2007; Fowler, 2008; Seifter *et al*., 2005). There is a direct clinical practice to link these complications with DM type 1 rather than type 2 (Retenakaran and Zinman, 2008). Of note, type 2 DM may manifest development of microvascular complication 7 years preceding diagnosis (King *et al*., 2005).

2.2 Macrovascular complications

These define the pathophysiology of cardiac disease in type 1 DM which includes atherosclerosis, coronary artery diseases, stroke and peripheral arterial disease (Retenakaran and Zinman, 2008; Seifter *et al*., 2005). These cardiovascular complications are mostly focused in clinical practice to type 2 DM (Fowler, 2008), and increased in rate as well in type 1 DM (Mcphee *et al*., 2008); however, the mortality impact of cardiovascular diseases in both DM types 1 and 2 is similar (Juutilainen *et al.,* 2008) contributing to 80 % of the mortality and morbidity (Coccheri, 2007). More alarmingly, development of macrovascular complication might not manifest in type 1 DM until 10 years proceeding diagnosis (King *et al.,* 2005).
Endothelial and smooth muscle dysfunction has been associated with type 1 diabetic patients, which in turn might cause hypertension, a major risk factor for developing cardiovascular diseases (Retenakaran and Zinman, 2008). Also lipid abnormalities in type 1 DM, decrease in HDL and increase in LDL compositions, are being incidents of causing cardiovascular diseases (Retenakaran and Zinman, 2008). High levels of ketone bodies are produced from the body as an alternate fuel in the absence of glucose as a main source of energy in severe starving conditions, mainly hyperglycemia (Seifter *et al*., 2005), or an acute multiplication of type 1 but not type 2 DM (Rhoades and Bell, 2009). Three types of ketones can be generated in the body these are acetone whose odor can be detected overtly *via* breath, acetacetic acid and β-hydroxybutyric acid whose levels may elevate in the blood and be excreted in urine, which is followed by cations and fluid loss ultimately leading to coma (Rhoades and Bell, 2009).

3. Renin angiotensin system

With the discovery of renin in 1898, Robert Tigerstedt and Per Bergman were able to fill a gap present in the understanding of fluid balance, hypertension and cardiovascular disease (Basso and Terragno, 2001**;** Phillips and Schmidt-Ott, 1999). The Renin Angiotensin System (RAS) plays an important role in maintaining normal blood volume and blood pressure. When salt and water intake is reduced, the role of RAS becomes critical (Rhoades and Bell, 2008). Upon low plasma volume, intrarenal baroreceptors found in the afferent arteriole

walls sense a decrease in stretch and neighboring granular cells increase renin synthesis and release (Boron and Boulpaep, 2009). The granular cells of the juxtaglomerular apparatus of the kidney produce renin (Widmaier *et al.,* 2004). At first, preprorenin is synthesized in the granular cells; its 23 amino acid signal peptide is then cleaved to form prorenin (Sepehrdad *et al*., 2007). This proenzyme undergoes further modification- clipping the 43 amino acid segment from the N-terminal – to produce renin (Pool, 2007). Despite the conversion of prorenin to renin, prorenin remains the predominant form in the systemic circulation and represents 90% of the total plasma renin in humans (Pimenta and Oparil, 2009). Renin belongs to the family of aspartyl proteases, including pepsin, cathepsin D and chymosin (Verdeccia *et al*., 2008). Unlike other aspartyl proteases however, renin is able to work at neutral pH. Moreover, renin is specific for one substrate only- angiotensinogen (Sepehrdad *et al*., 2007). Made up of two lobes with a cleft in between renin accommodates the liver-derived angiotensinogen in its active site, where a peptide bond of angiotensinogen is hydrolyzed and the decapeptide Angiotensin I (Ang I) is released (Verdeccia *et al*., 2008). Furthermore, this conversion is catalyzed 4-fold upon the binding of renin to its receptor (Pool, 2007). Angiotensin converting enzyme (ACE) is the principal enzyme that converts Ang I to the octapeptide Ang II (60% conversion); some other enzymes such as chymases, cathepsin G and other serine proteases account for the rest of the conversion (Weir, 2007).

3.1 Cross talk between RAS and litus

Angiotensin II was always considered in close proximity with diseases and vascular complications. This is implied from data linking high levels of Ang II to DM and vasoconstriction (causing hypertension) (Karam *et al*., 2005), in addition to glomerular damaging ending up with nephropathy (Coccheri, 2007). Other than its direct effect as a potent vasoconstrictor, it was denoted to alter Endothelin-1 (ET-1) production (Karam *et al*., 2005), remodel cardiovascular structure (Parmar and Jadav, 2007), modulate heart and vessels (Nuwayri-salti *et al*., 2007), augment transforming growth factor–beta (TGF-β) and boost proliferative and inflammatory events (Wiggins and Kelly, 2009). In conjunction; modulation of insulin significance is obtained (Karam *et al*., 2005), either by directly halting signaling mechanism of insulin per se or damaging structure and function of β-cells *via* local pancreatic RAS (Coccheri, 2007). Angiotensin II has 2 receptors: Ang II Type 1 receptor (AT1R) and Ang II Type 2 receptor (AT2R). But until now AT1R rather than AT2R is assessed in the pathophysiology (Karam *et al.*, 2005; Rao, 2010) directing therapeutic agents either to target Ang II formation or block its binding to AT1R *via* Angiotensin converting enzyme inhibitors (ACEIs) and Ang II Receptor Blocker(ARB) respectively (Karam *et al.,* 2005). AT1R blockers improved vascular smooth muscle cell vasoconstriction and declined the hypertrophy of cardiomyocytes (CM) counteracting Ang II deleterious effect (Al Jaroudi *et al*., 2005) once bound to the G-coupled protein receptor (AT1 R) (Parmar and Jadav, 2007; Wiggins and Kelly, 2009). Quite interestingly, ACEIs ameliorated insulin sensitivity and mitigated new onset of DM type 2 (Hadi and Suwaidi, 2007; Scheen, 2004). Pooled together, these G-coupled transmembrane proteins mimic insulin receptor action (Nuwayri-Salti *et al*., 2007). Among several evidences, AT2R is more related to apoptotic actions thereby antagonizing insulin's growth effect, resulting in one way or another to boost cardiac hypertrophy mainly in type 1 DM (Al Jaroudi *et al*., 2005). To complete this picture, a balance should be assured between both Ang II receptors to modulate a normal cardiac status in type 1 DM along the endothelial cells and cardiomyocytes (Al Jaroudi *et al*., 2005).

The idea of blocking RAS at its point of origin was initiated around 50 years ago (Pool, 2007). The origin of these direct renin inhibitors (DRIs) was either analogues of the prosegment peptide of renin or of the AGT's N-terminal amino acid sequence (Gradman and Kad, 2008). Many peptidomimetic synthetic drugs used for renin inhibition have been launched including pepsatitn (the first renin inhibtor) and other oral drugs, including remikiren, enalkiren and zankiren (Pimenta and Oparil, 2009), but their low efficiency related to large molecular size, first-pass metabolism, incomplete intestinal absorption, hydrophobicity and short half-lives attributed their poor oral activity and bioavailability, besides the high production cost (Pool, 2007; Waldmeier *et al*., 2007). The drug Tekturna® (United States) or Rasilez ® (Europe) , known with the generic name of Aliskiren, ascribed to Dr. Alice Huxley (Gradman and Kad, 2008), manufactured by Novartis Pharmaceuticals, was the first of the nonpeptide DRIs to be approved by the Food and Drug Administration (FDA) (Azizi, 2008). Thereby becoming the commercially available renin blocker in markets, prescribed through United States and Canada for an effective essential hypertension treatment in March 2007 (Azizi, 2008), and approved by European Medicine Agency (EMEA) in August 2007 (Pool, 2007; Azizi, 2008).

4. Endothelin system

In 1985, Hickey *et al.* discovered a potent vasoconstricting substance from cultured endothelial cells and named it endotensin or endothelial contracting factor. Later on, Yanagisawa *et al*. isolated the same substance from cultured porcine aortic and endothelial cells and renamed it endothelin. Endothelin (ET) is a naturally occurring polypeptide (Prasad *et al*., 2009) produced by the endothelium (Shah et al.,2009; Wikes et al., 2003; Ying wu, 2003) and consists of 21 amino acids. It is present in three isoforms ET-1, ET-2 and ET-3 (Agapitov *et al*., 2002; Prasad *et al*., 2009). These three isoforms are encoded by different genes but have similar structure and function (Wilkes *et al*., 2003). They have conserved sequences of amino acids mainly at the 6 C terminus and 4 cysteine residues that form 2 disulfide bridges between residues 1 to 15 and 3 to 11 and the main difference is at the N-terminus (Prasad *et al*., 2009). They are vasoconstrictors synthesized by vascular, right atrial and left ventricular endothelial cells, vascular smooth muscle cells and fibromyocytes. In addition, they are synthesized in extra vascular tissues such as the lungs, spleen, pancreas and nervous system (Penna *et al*., 2006). All studies done on endothelial systems focused on ET-1, because represents the majority of the circulating endothelins (Prasad *et al*., 2009; Schneider *et al*., 2002) and has important role in the regulation of the cardiovascular system (Penna *et al*., 2006; Prasad *et al*., 2009).

4.1 Endothelin-1

Endothelin-1 is the most common isoform correlated with the cardiovascular system (Kalani, 2008). It was identified in the late 1980s by Dr. Yanagisawa and his colleagues and found to be a very potent vasoconstrictor (Prasad *et al*., 2009; Steiner and Preston, 2008; Thorin and Webb, 2009). It has ionotropic, chemotactic and mitogenic activities. It influences salt and water retention through its effect on RAS, vasopressin release and stimulation of sympathetic nervous system. So the ultimate role of ET-1 is to increase blood pressure (Agapitov *et al*., 2002) and to maintain vascular tone (Thorin and Webb, 2009). It is produced mostly from endothelial cells in addition to fibroblasts, cardiacmyocytes (Agapitov *et al*.,

2002; Schorlemmer *et al*., 2008), leukocytes, macrophages (Thorin and Webb, 2009), kidney, central nervous system and posterior pituitary (Agapitov *et al*., 2002).
Endothelin-1 exerts a paracrine/autocrine effect. In the circulation, the levels of ET-1 are in picomolars lower than that needed to cause vasoconstriction (Prasad *et al.,* 2009). Also endothelial cells secrete more ET-1 toward the vicinity of vascular smooth muscle cells than into the lumen of the blood vessels (Agapitov *et al*., 2002).

4.2 Endothelin-1 synthesis and clearance

Endothelin-1 synthesis begins with the cleavage of a 200 amino acid peptide called pre-proendothelin-1 (preproET-1) to form preendothelin-1 (preET-1) (Agapitov *et al.,* 2002; Penna *et al*., 2006). PreET-1 is then cleaved by furin endopeptidase to form big endothelin-1 (big ET-1) of 38-39 amino acids that is further metabolized by Endothelin Converting Enzyme-1 (ECE-1) to generate ET-1 of 21 amino acids (Agapitov *et al*., 2002; Penna *et al*., 2006). Moreover, chymase enzyme was found to produce ET(1-31) by breaking down big ET-1 at Tyr31-Gly32 bond (Agapitov *et al*., 2002).
Furthermore, the regulation of ET-1 synthesis is at the gene level which means at the level of ET-1 messenger RNA (mRNA). The ET-1 mRNA is up-regulated by several factors including: interleukins, insulin, Ang II, tumor necrosis factor alpha and growth factor while it is down regulated by hypoxia, shear stress and nitric oxide (Thorin and Webb, 2009). The clearance of ET-1 from the plasma involves different mechanisms including: (1) endocytosis in the lungs, (2) enzymatic degradation in the liver and the kidney and (3) ET-1 binding to ET_B receptor thus forming ET_B Receptor-Legand complex that is broken down by endocytosis (Kalani, 2008).

4.3 Cross talk between endothelin system and renin angiotensin system

The Renin Angiotensin and the Endothelin Systems are the most potent identified vasoconstrictory systems and has been suggested to be correlated with each other (Rossi *et al*., 1999; McEwan *et al*., 2000). This was demonstrated by several cell culture studies in cardiac fibroblasts. These studies showed that AngII increases preproET-1 mRNA expression, ET-1 levels and thus causes cardiomyocyte hypertrophy through regulation of ET_AR. Also increased levels of ET_BR via Ang II have also been identified in cultured CM (McEwan *et al.,* 2000).
The potential sites at which RAS increases ET-1 levels may be at the preproET-1 gene that possesses a *jun* sequence which is defined as the regulatory region of preproET-1. It is the site at which transcriptional regulation takes place by factors acting through G-protein-phospholipase and C-protein kinase C pathway. Another site for interaction of RAS and ET system is at the chymase enzyme which is abundant in the myocardium where it was found to produce ET (1-31) from big ETs**,** bearing in mind that the main function of chymase enzyme in the heart is to produce Ang II from Ang I (Rossi *et al*., 1999). Therefore, elevated levels of ET-1 can be reduced by blocking the RAS system. Studies on patients suffering from hypertension or diabetes showed decrease in the level of ET-1 when ACEI was administered (Schneider *et al*., 2002).
Therapies targeting these complications include the use of ARBs which proved their value as antihypertensive drugs. Losartan, an ARB, was found to decrease the destructive changes caused by Ang II on cardiac muscle beside its (Berk, 1999; Fiordaliso *et al*., 2000) capacity to reduce blood pressure. Recently, Al Jaroudi *et al*. proved that losartan in diabetic normotensive rats supplemented with insulin was able to prevent nearly totally myocardial degeneration caused by diabetes (Al Jaroudi *et al*., 2005) (Fig. 1).

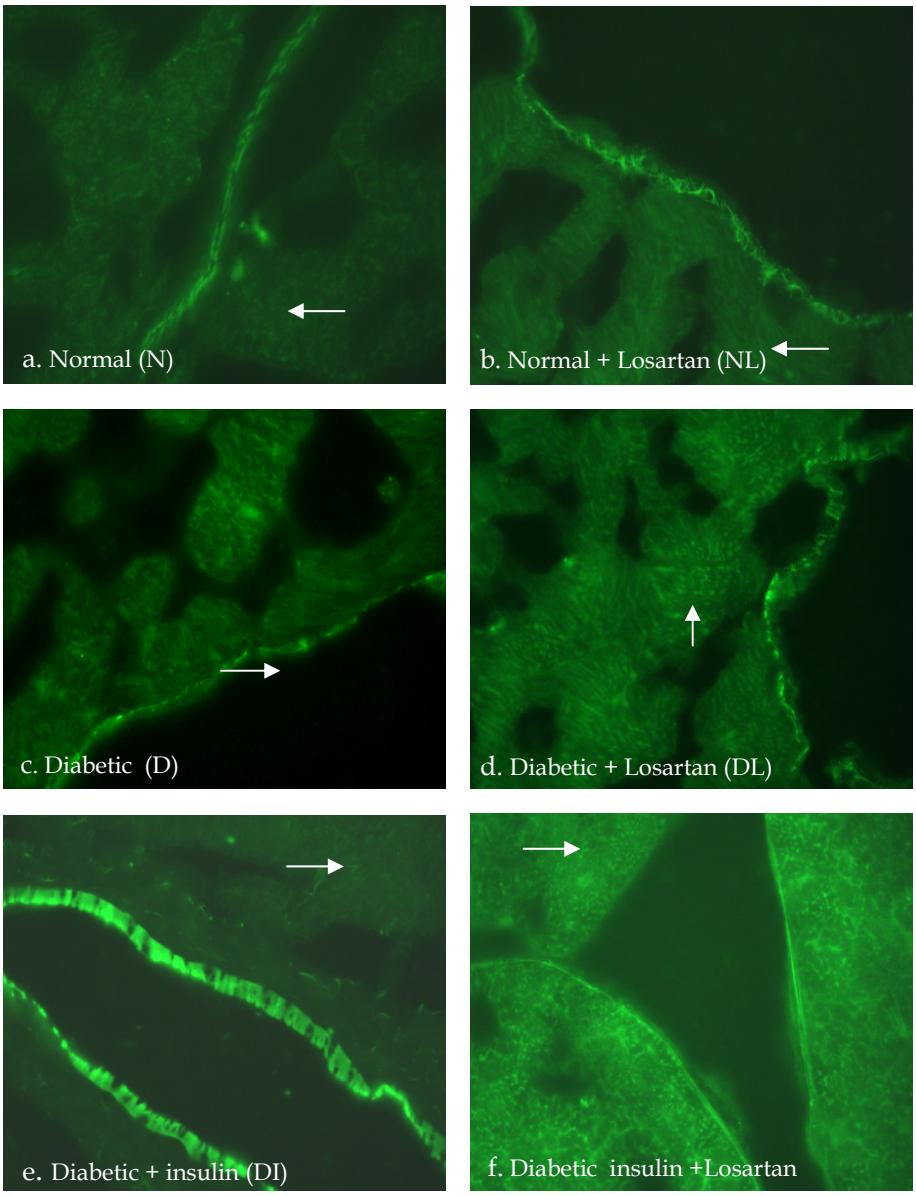

Fig. 1. Indirect immunofluorescence of CM and vascular endothelium from the different rat groups depicting insulin receptors. Noted is the increase of fluorescence in the NL group (Fig. b) when compared to the normal (N) (Fig. a) at the myocytes (arrow). There is also increase fluorescence of the myocytes of the (DL) group as compared to the (D) group, and of the (DIL) group as compared to the (DI) group (arrow).

Later, Karam *et al.* reported that the supplementation with insulin and administration of pharmacologic doses of losartan could improve cardiac contraction as well as coronary blood flow in the same normotensive rat model for type 1 DM as the one used by Jaroudi *et al.* through the modification of the affinity of ET type 1 receptor subtypes ET_AR and ET_BR to ET-1, a potent vasoconstrictor largely stimulated by Ang II (Karam *et al.*, 2005). In fact, Kakoki *et al.* have demonstrated that the ET_BRs are down-regulated by Ang II in the endothelial cells of the renal artery of diabetic rats through stimulation of increased ET-1 production (Kakoki *et al.*, 1999). On the other hand, these effects of Ang II are prevented by the ACE inhibitor Imidapril (Kakoki *et al.*, 1999). ACE inhibitors reduced also the plasma ET-1 levels in type 1 DM (Schneider *et al.*, 2002). Administration of Losartan to Ang II-treated rats restored the vasoconstrictive effect of ET-1 and decreased its tissue levels (D'Uscio *et al.*, 1997). Mc Ewan *et al.* showed that the blockade of the AT1Rs by Losartan, in the presence of a high plasma Ang II levels, is accompanied by an increase in ET-1 production by the CM of Ang II-treated rats.
In addition to this, an increase in the ET_BRs mRNA but a decrease in the ET_ARs mRNA was also noted (Mc Ewan *et al.*, 2000). On the other hand, treatment with the selective ET_AR blocker LU135252 normalized the increased ET-1 level in the aorta, femoral artery and kidney, and the ECE activity in isolated aorta and femoral artery of rats treated with Ang II (Barton *et al.*, 1997). In cultured heart endothelial cells, Ang II stimulates ppET-1 mRNA and ET-1 production via Protein Kinase C (PKC) - dependent pathway. Calphostin, a PKC inhibitor, blocks Ang II effects (Chua *et al.*, 1993). Taken together, these results show that the ET and the RA systems are related to each other. They also demonstrate the beneficial role of ARBs and ACE inhibitors in the amelioration of the blood flow which can be altered in some disease states due to the ET-1 and Ang II constrictor effects.

5. Insulin

The discovery of insulin in 1921 was one of the most dramatic events in the history of diabetes therapy. Insulin is a small globular protein comprising of two polypeptide chains A (21 amino acid residues) and B (30 amino acid residues) held together by two disulfide bonds (Docherty and Steiner. 2003; Joshi *et al*. 2007). It is produced in the β-cells of the islets of Langerhans in the form of preproinsulin which is rapidly cleaved by the action of proteolytic enzymes into proinsulin. Further catalysis of proinsulin results in insulin and a 31 amino acid connecting peptide, C-peptide, both of which are stored for secretion in secretory vesicles in β-cells (Docherty and Steiner. 2003). The biosynthesis and secretion of insulin by β-cells primarily occurs in response to increased circulating glucose levels. During feeding, elevated glucose concentrations in the blood increase the plasma insulin level, which facilitates glucose uptake through GLUT-4 into muscle tissues for utilization as a source of energy and into adipose tissues for synthesis of glycerol. Insulin also exerts its action on liver cells as well thus promoting glycogen formation. Consequently, glucose utilization by these different tissues contribute to the decrease in the concentration of glucose in blood. On top of its profound effect in carbohydrate metabolism, insulin has a fat-sparing effect. Not only does it promote the synthesis of fatty acids in the liver, it also inhibits the breakdown of fats in adipose tissue thereby inducing fat accumulation. Hence, insulin is the major determinant of carbohydrate and lipid metabolism and has significant effects on protein metabolism. Insulin acts by binding to a receptor molecule embedded in the plasma membrane of its target tissues (Docherty and Steiner. 2003).

5.1 Insulin receptor

Insulin receptor is a member of the receptor tyrosine kinase family (De Meyts and Whittaker, 2002; Kanzaki, 2006; Klarlund *et al*., 2003; Lawrence *et al*., 2007; Stern, 1995; Ward *et al*., 2008). It is a large cell-surface multi-domain glycoprotein that consists of two extracellular α-subunits (MW~125 kDa) and two transmembrane β-subunits (MW~95 kDa) (Kanzaki, 2006; Klarlund *et al*., 2003; Lawrence *et al*., 2007; Stern, 1995; Ward *et al*., 2008) linked by disulfide bonds (Kanzaki, 2006; Lawrence *et al*., 2007) into an $\alpha_2\beta_2$ heterotetrameric complex (Kanzaki, 2006). Extracellular α-subunits contain the insulin-binding site (Klarlund *et al*., 2003) while intracellularly transmembrane β-subunits contain the insulin-regulated tyrosine kinase catalytic domain (De Meyts and Whittaker, 2002; Klarlund *et al*., 2003; Ward *et al*., 2008). Two nearly identical isoforms (A and B) of the insulin receptor exist due to tissue specific alternative splicing of the receptor m-RNA at exon 11. Isoforms A and B differ by the presence or absence of a 12 amino acid sequence at the carboxyl-terminus of the α-subunit (Anderson *et al*. 1993; De Meyts and Whittaker. 2002; Klarlund *et al*., 2003; Lawrence *et al*., 2007). Despite the known biochemical and physiologic differences of these isoforms, changes in their expression levels have not been consistently found in patients with DM (Klarlund *et al*., 2003). Once insulin binds to the α-subunit of the insulin receptor, it induces a conformational change in the receptor that subsequently leads to the stimulation of the intrinsic tyrosine kinase activity in the β-subunits (Kanzaki, 2006; Klarlund *et al*., 2003; Stern, 1995). This results in the transfer of phosphate groups from ATP to several tyrosine residues on the insulin receptor itself as well as phosphorylation of cellular proteins such as insulin receptor substrate-1 (IRS-1) and Shc (Klarlund *et al*., 2003; Stern, 1995). The stimulation of the receptor tyrosine kinase allows transmission of the insulin signal to metabolic pathways such as glucose uptake upon translocation of GLUT-4 glucose transporters to the plasma membrane, glycogen synthesis, protein synthesis, and lipid metabolism within the cell. The various biological responses generated upon insulin receptor activation through insulin binding granted insulin a role in DM treatment due to its glucose lowering effects. Nevertheless, daily insulin injections continue to be a treatment for diabetic patients since 1922 despite the increasing worldwide prevalence of this disease (Sparre *et al*., 2005). Furthermore, insulin treatment dramatically prolongs survival, but does not cure diabetes (Myers and Zonszein, 2002). Insulin treatment does not seem to be beneficial for many patients and is associated with weight gain, hypoglycemia, and failure of their glycemic control (Halimi, 2008). Therefore, new treatment modalities are intensively studied mainly the incretins, exemplified by GLP-1.

6. Definition of incretins

The intravenous infusion of glucose at a constant rate results in a biphasic insulin secretory response, in which the first peak rises rapidly followed by a slower second peak. In contrast, an oral administration of glucose followed by its gastrointestinal absorption, triggers a hormonal pathway that eventually leads to a far greater response of insulin secretion which can last as long as glucose is administered. This phenomenon is termed as the 'Incretin Effect' (Rhoades and Bell, 2008).

Incretins are hormones that are secreted by the gut upon feeding; their release alerts the pancreatic islets that nutrients will come through the gastrointestinal tract and islets start priming by vagal stimulation (Boron and Boulpaep, 2009). Glucagon-Like Peptide-1 (GLP-1) and Glucose Dependent Insulinotropic Peptide (GIP) are the major incretin hormones

produced by the L cells of the ileum and colon, and K cells of the duodenum and jejunum, respectively (Inzucchi and McGuire, 2008).

6.1 Synthesis of glucagon-like peptide-1

The proglucagon gene, located on chromosome 2 in humans, has the coding sequence of GLP-1. Pancreatic α-cells, intestinal L-cells and neural cells in the caudal brainstem and hypothalamus, express the proglucagon gene (Baggio and Drucker, 2007) and the mRNAs produced in the pancreas and intestine are identical. Its post-translational processing, however, differs in the two tissues. The post translational processing of proglucagon in pancreatic α- cells results in Glucagon, Glicentin-Related Pancreatic Polypeptide (GRPP), Intervening peptide-1 (IP-1) and the major proglucagon fragment (MPGF) (Holst, 2007). The latter codes for the production of GLP-1 (1-36 amide) and GLP-1 (1-37) and GLP-2 in the pancreas (Hui *et al*., 2005). Whereas the intestinal L and brain cells produce GLP-1 (7-36 amide), GLP-1 (7-37), GLP-2, IP-2, and glicentin that if further cleaved produces GRPP and oxyntomodulin (Holst, 2007). The enzyme prohormone convertase (PC) 1/3 cleaves proglucagon to generate GLP-1 (Shin *et al*., 2008). GLP-1 is an incretin hormone produced primarily by the L-cells in the mucosa of the ileum and the colon (Mannucci and Rotella, 2008). Small neurons found in the Nucleus Tractus Solitarius (NTS), caudal brainstem, also produce GLP-1 that plays the role of a neuromodulator (Berthoud, 2009). The central nucleus of the amygdala (CeA) and the paraventricular nucleus of the hypothalamus (PVN) are also sites of GLP-1 production (Kinzig *et al.,* 2002).

Recently, GLP-1 production was shown to exist in taste bud cells (Shin *et al.,* 2008; Berthoud, 2009) and in particular in α-gustducin and the sweet taste receptor subunit T1R3, that play an important role in mediating the glucose dependent secretion of GLP-1 (Shin *et al*., 2008**)**. GLP-1 is usually present in the circulation minutes after meal intake- far before food reaches the L-cells in the gut. Hence, its stimulation is believed to be controlled by both endocrine and neural signals (Drucker, 2007). The two active forms of GLP-1 are the 30 amino acid GLP-1 (7-36) amide and the 31 amino acid glycine extended GLP-1 (7-37) (Ban *et al.,* 2009; Mannucci and Rotella, 2008). GLP-1 (7-36) amide is far more abundant than GLP-1 (7-37) in the circulation (Manucci and Rotella, 2008), but both have short half-lives (1.5-2 minutes) (Hui *et al*., 2002). They are rapidly degraded into their inactive forms, GLP-1(9-36) amide and GLP-1 (9-37), respectively, by the enzyme Dipeptidyl-Peptidase-IV (DPP-IV) (Mannucci and Rotella, 2008) and eliminated through renal clearance (Hui *et al.,* 2002).

6.2 Effects of GLP-1

GLP-1 has numerous effects on glucose homeostasis. Upon interaction with its receptor on β-cells, GLP-1 increases the intracellular levels of cAMP and calcium, thereby releasing insulin (Drucker, 2007). Furthermore, sustained GLP-1 receptor (GLP-1R) signaling leads to enhanced gene transcription, insulin biosynthesis and β-cell proliferation (Elahi *et al.,* 2008; Drucker, 2007). GLP-1 was shown to increase the expression of glucose transporter 2 (GLUT2) (Elahi *et al.,* 2008), a hepatic glucose transporter that facilitates glucose transport in or out of the liver regulated by insulin (Eisenberg *et al*., 2005) and glucokinase (Elahi *et al*., 2008) the enzyme that phosphorylates glucose to glucose-6-phosphate (G6P) (Voet and Voet, 2004). GLP-1 has also inhibitory effects on Glucagon secretion from the α-cells, gastric emptying and food ingestion (Drucker, 2007). The role of GLP-1 in relaxing the proximal stomach and increasing gastric capacity has been demonstrated (D'Alessio and Vahl, 2004).

GLP-1 also exerts effects on the central nervous system: it promotes satiety and weight loss, inhibits food and water intake and improves memory and neuronal survival (Mannucci and Rotella, 2008; Drucker, 2007).

6.3 GLP-1 receptor

The GLP-1R belongs to the Guanine Nucleotide-Binding Protein (G-protein) coupled receptor family (GPCR) (Drucker, 2007). This seven transmembrane receptor protein is 90% identical to the rat GLP-1R and shows a 95% homology in amino acid. Its gene is found on the long arm of chromosome 6p21 in humans (Doyle and Egan, 2007). GLP-1 receptor is made up of 463 amino acid residues and has a molecular weight of approximately 65 kDa (Hui *et al*., 2005). GLP-1 receptors are expressed in many tissues including, the central and peripheral nervous systems, heart, kidney, lungs and the gastrointestinal tract (Drucker and Nauk, 2006).

However, GLP-1 receptor expression in the pancreas is controversial. Studies have confirmed the detection of GLP-1 receptors in α, β and δ cells of the islets of Langerhans, whereas other studies indicate their expression exclusively in β cells (Doyle and Egan, 2007). In the heart, GLP-1 receptor expression was shown to exist in CM, microvascular endothelium, coronary smooth muscle cells and the highest in endocardium. In cardiac fibroblasts, however, there was no evidence in GLP-1R expression (Ban *et al*., 2009). Low detection of GLP-1 receptor gene expression proves the existence of GLP-1 receptor in liver and muscles (MacDonald *et al*., 2002). Abundant GLP-1R specific transcripts were found in lungs and detectable amounts in the heart (Ban *et al*., 2009). When fragments of the N-terminus are denatured, GLP-1R loses its affinity for GLP-1. This asserts that GLP-1 receptor's N-terminus plays an important role in recognizing and binding of the GLP-1 (Doyle and Egan. 2007). Upon binding of the GLP-1 to its receptor numerous signaling messengers are activated; first, GLP-1 receptor can couple to G proteins, including $G\alpha_s$, $G\alpha_q$, $G\alpha_i$ or $G\alpha_o$ (Baggio and Drucker, 2007). Adenylate cyclase uses ATP to form cAMP via the stimulatory G protein. Following the increase of cAMP is a chain of events is triggered: ATP-sensitive K^+ channels shut and L-type voltage gated Ca^{2+} channels open; together with the efflux of Ca^{2+} molecules from intracellular Ca^{2+} stores, these ion channel alterations result in a subsequent intracellular rise in Ca^{2+} ions (Gomez *et al*., 2002) and the ultimate exocytosis of granules that contain insulin. A sustained receptor signaling results in the activation of Protein Kinase A (PKA), gene transcription, insulin biosynthesis and β-cell proliferation (Drucker and Nauk, 2006). Additionally, the binding of GLP-1 to its receptor activates protein kinase B, which is linked to glucose transporting in muscles, glycogen synthesis and lipolysis in various tissues (Peyot *et al*., 2009).

6.4 GLP-1 in diabetes mellitus and the heart

A recent study showed that GLP-1 receptor expression is downregulated in β-cells exposed to high glucose concentrations in vitro and hyperglycemia in vivo (Xu *et al*., 2007). Diabetic individuals' β-cells exhibit attenuated sensitivity to GLP-1. In both type 1 and type 2 DM, there is a marked reduction in the incretin effect (Knop *et al*., 2007). On the other hand, a study reported that GLP-1 levels are not decreased in type 2 diabetic patients (Lee *et al*., 2010). The European GLP-1 Club held a meeting recently and debated this controversial issue and came to a conclusion that more data is required to determine the exact effect of DM on GLP-1 secretion from L-cells (Burcelin, 2008). GLP-1 has been suggested to

ameliorate left ventricular function, because of its antiapoptotic and insulin-like properties (Inzucchi and McGuire, 2008). In fact, one study confirmed that GLP-1 enhances the regulation of phosphatydil inositol 3 kinase (PI3K), that plays a key role in activating the antiapoptotic pathway, thus promoting cardioprotection in the ischemic rat hearts. Therefore, a direct effect of GLP-1 against apoptosis in cardiac cells is possible (Bose *et al*., 2005).

6.5 Dipeptidyl-peptidase IV

DPP-IV grasped a great deal of the interest of the scientific, pharmaceutical, and medical community (Lambeir *et al*., 2003). Every year, an increasing number of publications tend to elucidate the diverse compelling questions concerning the various properties of DPP-IV and its multiple functions in the different fields of Biology (Lambeir *et al*., 2003). DPP-IV is a 766 amino acid serine protease that belongs to the prolyloligopeptidase family. It is a widely distributed cell surface peptidase expressed to different degrees in a variety of tissues such as the kidney, intestine, liver, placenta, uterus, prostate, skin, and capillary endothelium. Another soluble form of DPP-IV (sDPP-IV) also exists in the plasma and other body fluids (Drucker, 2007; Idris and Donnelly, 2007; Lambeir *et al*., 2003). This proteolytic enzyme acts by specifically cleaving the N-terminal dipeptide of peptide hormones containing proline or alanine in the second position (Drucker, 2007; Idris and Donnelly, 2007; Lambeir *et al*., 2003). Hence, DPP-IV truncates several biologically active peptides of medical importance. Furthermore, it has been implicated in glucose homeostasis through proteolytic degradation of the incretins (Drucker, 2007). In the case of GLP-1(7-36), proline and alanine are key determinants in incretin receptor activation therefore DPP-IV-mediated proteolysis results in the biologically inactive truncated molecules GLP-1(9-36) (Drucker, 2007; Idris and Donnelly, 2007). DPP-IV is a critical determinant of incretin inactivation (Drucker, 2007). Thus chemical inhibition of DPP-IV activity results in increased level of biologically active GLP-1 (Drucker, 2007). Therefore, extensive research studies were carried to create highly selective DPP-IV inhibitors.

6.6 DPP-IV inhibitors

DPP-IV inhibitors can be used as a potential treatment for diabetes due to their capability to potentiate the levels of active incretins (Lambeir *et al*., 2003; Halimi, 2008; Inzucchi and McGuire, 2008) by reducing the enzymatic activity of DPP-IV by more than 80% for duration up to 24 hours (Inzucchi and McGuire, 2008). Oral inhibitors of DPP-IV reduce glycosylated hemoglobin (HbA_{1C}) (Drucker, 2007; Halimi, 2008; Moritoh *et al*., 2008). Various studies reported the multiple metabolic effects of DPP-IV inhibitors including enhancement of glucose-dependent stimulation of pancreatic insulin release as well as attenuation of glucagon secretion (Drucker, 2007; Halimi, 2008; Lambeir *et al*., 2003; Moritoh *et al*., 2008). Furthermore, DPP-IV inhibitors demonstrated modestly effective glucose-lowering actions without being associated with hypoglycemia (Halimi, 2008; Lambeir *et al*., 2003; Moritoh *et al*., 2008) due to the fact that they are remarkably able to specifically end the insulin-secreting effect and glucagon inhibition once glycemic levels are normalized (Halimi, 2008). Moreover, loss of DPP-IV activity is associated with improved glucose tolerance, reduced glycemic excursion following oral glucose challenge, and increased pancreatic insulin content (Drucker, 2007). A cardinal role of DPP-IV inhibitors is their potential to significantly augment β-cell mass in streptozotocin (STZ)-injected diabetic rats (Moritoh *et*

al., 2008) and enhance pancreatic islet cell function in animal models of type 2 diabetes and in diabetic patients (Lambeir *et al*., 2003). In contrast to GLP-1 mimetics, there are no data indicating inhibition of gastric emptying or appetite or weight reduction due to a treatment with DPP-IV inhibitors (Inzucchi and McGuire, 2008). Thus, chronic treatment with orally administered DPP-IV inhibitors has neutral effects on body weight and food consumption (Halimi, 2008).

6.7 GLP-1 receptor agonists and GLP-1 analogues

In April 2005, the FDA approved the subcutaneous injectable exenatide with the brand name Byetta® to be launched as a GLP-1 analogue (Inzucchi and McGuire, 2008; Rhoades and Bell, 2009) with longer half life to maintain its glycemic control as diabetic medication (Behme *et al*., 2003). Exenatide exhibits a half-life of 60-90 minutes and a single injection of exenatide makes its concentration in the blood last for 4-6 hours (Drucker and Nauk, 2006). Exenatide exhibits antidiabetic effects similar to those of GLP-1 and is shown to improve β cell functioning (Baggio and Drucker, 2007). It also reduces the glycated Hemoglobin-HbA1c- levels that reflect mean blood glucose levels of the last 6-8 weeks. In addition, exenatide affects gastric emptying, appetite and consequently causes weight loss (Inzucchi and McGuire, 2008). GLP-1 agonists have exhibited vasodilating and diuretic effects. Recently, Laugero *et al.* showed that Exenatide may display antihypertensive effects mediated by pathways independent from glucose control, but possibly by altering steroid hormones (Laugero *et al*., 2009).

6.7.1 GLP-1 analogue, exendin-4

The short half-life of GLP-1 limits itself from performing as a good therapeutic agent, as it is rapidly degraded by the serine protease DPP-IV (Hirata *et al.,* 2009). The search for biologically active peptides in lizard venom, led to the discovery of Exendin-4, a naturally occurring GLP-1 analogue (Drucker and Nauk, 2006). Exendin-4 is a peptide naturally found in the saliva of heloderma suspectum - the Gila monster (Laugero *et al*., 2009; Zhou *et al.,* 2008). Exendin-4 is a peptide made up of 39 amino acid residues that shares a 53% structural homology to GLP-1 (Zhou *et al.,* 2008).This long acting GLP-1 receptor agonist has an N-terminus almost identical to that of the GLP-1, except that the second amino acid residue is glycine in exendin-4 while alanine in GLP-1 (Hui *et al.,* 2005). This one amino acid difference makes it resistant to degradation by the enzyme DPP-IV, hence explaining its long half-life in vivo (Lovshin and Drucker, 2009). Studies have shown that GLP-1 receptor agonists like exendin-4, exhibit cardioprotective effects such as modifications in contractility, cardiac output and blood pressure (Ban *et al.,* 2009).

6.7.2 Mode of action of exendin-4

Endogenous GLP-1 action is inhibited upon degradation by DPP-IV (De Koning *et al*., 2008; Mann *et al*., 2007), an enzyme expressed in many organs mainly kidney, small intestine, liver, and lung (Inzucchi and McGuire, 2008). More apt, exendin-4 is synthetic, thus not recognized by DPP-IV enzyme, thereby not degraded (Rhoades and Bell, 2009). This resistance allows it to stay in the circulation longer mimicking GLP-1 role in bolstering insulin secretion but with 5 to 10 times more powerful insulinotrpic effect (Hantouche *et al*., 2010; Xu *et al*., 1999). Moreover, it binds to GLP-1R with high affinity even after truncation of the first 8 amino acids at the N-terminal of the peptide unlike the endogenous GLP-1 (Mann

et al., 2007). GLP-1R is a G-coupled transmembrane receptor (GPCR) that is found in many organs, most importantly the pancreatic β-cells and ducts, heart, lung, kidneys (Ban *et al*., 2008), brain and stomach (Hantouche *et al*., 2010). Conceptual understanding of this receptor delineates its advantages in cardiac management (Ban *et al*., 2008). On the initiation of treatment, slight adverse effects might arise as nausea and vomiting (Inzucchi and McGuire, 2008), which are dose-dependent and will vanish with time (Mcphee *et al*., 2008). It can be used efficiently in type 1 DM (Behme *et al*., 2003) in conjunction with insulin irrespective of hypoglycemic effects attributed to its glucose-dependent action; in spite of insulin low efficiency with type 2 DM (Inzucchi and McGuire, 2008).

6.8 Exendin-4 in the treatment of diabetes mellitus correlated with cardiovascular risk

In conjunction to insulinotropic action of GLP-1 role, exendin-4 also mimics the action of insulin itself, through reinforcing the heart to uptake glucose irrespective of insulin level in both diabetic and non-diabetic cases (Hantouche *et al*., 2010) STZ-induced diabetic rats acquire cardiomyopathic damage on day 1 starting with apoptosis and ending with hypertrophied hearts (Al Jaroudi *et al*., 2005), particularly myocardial atrophy (Poornima *et al*., 2006). Thereby, associated with its glucose lowering effect, certain data emphasized a potential benefit of exendin-4 in recovery of heart failure and function of left ventricle (Inzucchi and McGuire, 2008). Just similar to that of GLP-1 in improving ventricular contractions (Hantouche *et al*., 2010) upon activation of pancreatic GLP-1R to boost insulin synthesis and secretion (De Koning *et al*., 2008). Treatment with exendin-4 was concomitant with no cardiovascular complications, inexorable renal progression, or escalating plasma lipid (De Fronzo *et al*., 2005). In type 2 DM exendin-4 has completed a novel fruition in the arena of organ rejuvenation especially at the level of both α and β cells mass, implementing a new born anti-hyperglycemic drug for solving the diabetes conundrum (Xu *et al*., 1999). This increase in pancreatic mass happens as a post hoc to β-cells neogenesis from either existing duct cells and/or replication of the already present β-cells (hyperplasia) and not from hypertrophy (escalating cell size) (De Koning *et al*., 2008). Pooled together, this regimen was found to be concomitant with cardioprotection, judicious vascular and cardiac actions based on the fact that endogenous GLP-1 has the same sequence in humans, rats and mice (Ban *et al*., 2008). Recently, it was found that exendin-4 ameliorates the sensitivity of insulin receptors towards insulin at both the CM and coronary endothelium (CE) level (Hantouche *et al*., 2010). This effect is achieved by either enhancing the sensitivity of insulin towards insulin or augmenting β-cells to release more of the hypoglycemic hormone (De Koning *et al*., 2008). Valuable efficacy of treatment with both exendin-4 and insulin alleviates cardiomypathic regressions associated in type 1 DM at the level of receptor affinity enhancement and insulin secretion bolstering (Hantouche *et al*., 2010) (Fig. 1 and 2).

Insulin affinity (τ) to its receptor was shown to be decreased in the diabetic state at the level of the CE as compared to normal. Exendin-4 treatment increases insulin receptor affinity at the CE in both diabetic and normal rat groups; whereas insulin tends to normalize the affinity irrespective of exendin-4 absence or presence (Table 1). Exendin-4 treatment probably augments insulin receptor affinity both in normal and diabetic state through exerting its insulinotropic actions (Nikolaidis *et al*., 2005) and/or by improving insulin sensitivity (Ebinger *et al*., 2000). The dramatic increase in the affinity of insulin to its receptor in the normal group treated with exendin-4 might be attributed to both of the previously mentioned causes. Yet the rise in insulin receptor affinity in exendin-4-treated diabetic group is not as remarkable as in NE which may be due to the lack of β-cells in diabetic type 1 state thus abolishing the insulinotropic effect of exendin-4.

Rat Group	**τ (min) ± SEM**
Normal (N)	0.279 ± 0.004 $^{a'}$
Normal + Exendin-4 (NE)	0.370 ± 0.007 $^{b'}$
Normal + DPP-IV inhibitor (N-Dp)	0.311 ± 0.005 $^{c'}$
Diabetic (D)	0.252 ± 0.003 $^{d'}$
Diabetic + Insulin (DI)	0.292 ± 0.004 $^{e'}$
Diabetic + Exendin-4 (DE)	0.327 ± 0.005 $^{f'}$
Diabetic + Insulin + Exendin-4 (DIE)	0.295 ± 0.004 $^{g'}$
Diabetic + DPP-IV inhibitor (D-Dp)	0.255 ± 0.003 $^{h'}$
Diabetic + Insulin + DPP-IV inhibitor (DI-Dp)	0.321 ± 0.005 $^{i'}$

Table 1. The calculated affinity constant (τ) of insulin to its receptor at CE (CHAPS-untreated) in normal and diabetic rats

Rat Group	**τ (min) ± SEM**
Normal (N)	0.337 ± 0.006 $^{a'}$
Normal + Exendin-4 (NE)	0.424 ± 0.009 $^{b'}$
Normal + DPP-IV inhibitor (N-Dp)	0.234 ± 0.003 $^{c'}$
Diabetic (D)	0.368 ± 0.007 $^{d'}$
Diabetic + Insulin (DI)	0.331± 0.006 $^{e'}$
Diabetic + Exendin-4 (DE)	0.328 ± 0.005 $^{f'}$
Diabetic + Insulin + Exendin-4 (DIE)	0.331 ± 0.006 $^{g'}$
Diabetic + DPP-IV inhibitor (D-Dp)	0.376 ± 0.007 $^{h'}$
Diabetic + Insulin + DPP-IV inhibitor (DI-Dp)	0.382 ± 0.007 $^{i'}$

Table 2. The calculated affinity constant (τ) of insulin to its receptor at CM (CHAPS-treated) in normal and diabetic rats.

Therefore, in diabetes type 1, exendin-4 increases the insulin receptor affinity only by improving insulin sensitivity. As a conclusion, exendin-4 seems to have supplementary effects which might explain the increase in τ value at the level of the CE. First, it augments insulin release from pancreatic β-cells when present in the normal state. This is its insulinotropic effect. Second, it improves insulin sensitivity possibly by inducing a conformational change in the insulin receptor (Ebinger *et al*., 2000). On the other hand, DPP-IV inhibitor (KR-62436, Sigma Chemical Company) treatment solely does not seem to be implicated in modulating insulin receptor affinity at the endothelial site in diabetic rats treated with DPP-IV inhibitor as compared to diabetics. Yet the effect of DPP-IV inhibitor at the CE becomes obvious in the presence of insulin, as seen in the diabetic group co-treated with insulin and DPP-IV inhibitor (DI-Dp) compared to diabetics (D) and in the normal group treated with DPP-IV inhibitor (N-Dp) with respect to normal. This could be due to some kind of a cross-talk between insulin and DPP-IV inhibitor which results in an increase of τ value in both N-Dp and DI-Dp. Moreover, insulin receptor affinity is not altered at the level of the CM in diabetics treated with DPP-IV inhibitor (D-Dp) and/or insulin (DI-Dp) when compared to diabetic rats (D) (Table 2). The major difference between the CE and CM

upon DPP-IV inhibitor treatment could be attributed to the fact that the already limited increase of GLP-1 by DPP-IV inhibitor is primarily imposed on endothelial cells which are the first site of encounter with systemic GLP-1. The difference in affinities observed between DPP-IV inhibitor and exendin-4 treatment might be due to the fact that DPP-IV inhibitor increases the systemically available GLP-1 levels; whereas exendin-4, besides its quantitative systemic effect, has a higher physiologic quality in term of being 5- to 10- fold more potent than GLP-1 (Saraceni and Broderick, 2007).

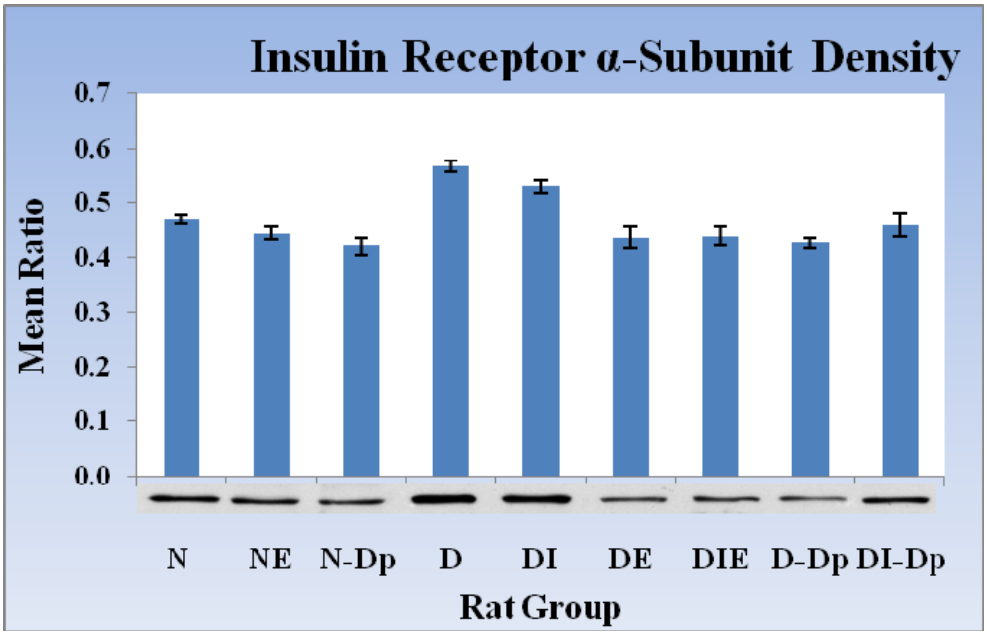

Fig. 2. Insulin receptor α-subunit (MW~125 kDa) density in the heart of the different rat groups.

Western blotting was performed on protein extracts from rat heart homogenates in order to assess the variation in the insulin receptor subunits density among the different treated and untreated normal and diabetic rat groups. Both insulin receptor α-subunit (IR-α) (Fig. 2) and insulin receptor β-subunit (IR-β) (Fig. 3) levels are augmented in diabetic state indicating that there is an increase in the level of cardiac insulin receptor protein in diabetics (D) compared with normal controls (N). These results indicate that cardiac insulin receptors are up-regulated in the heart of diabetic rats as a feedback mechanism probably due to the lack of insulin. This is consistent with the results demonstrated by Al Jaroudi *et al*. 2005 in the study done on insulin receptor regulation in the diabetic heart. Our results indicate that insulin administration to diabetic rats reduces the number of IR-α and IR-β to near normal values. Interestingly, exendin-4 treatment normalizes insulin receptor subunits density in diabetic rat hearts (Fig. 2 and 3). The regression in insulin receptor density with exendin-4 treatment is suggested to be attributable to the insulinomimetic effects of exendin-4 (Sokos *et al*., 2006) which result in a cross-talk between GLP-1 and insulin signaling pathways (Ebinger *et al*., 2000).

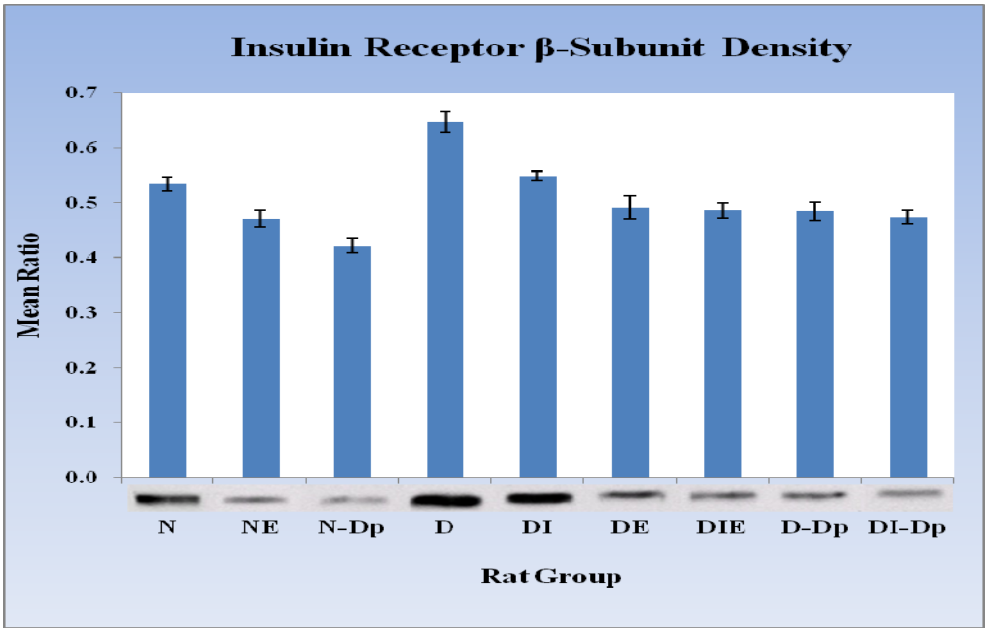

Fig. 3. Insulin receptor β-subunit (MW~95 kDa) density in the heart of the different rat groups.

There is no further attenuation in the level of insulin receptor upon insulin administration in combination with exendin-4 in diabetic rat groups. Thus, it is conceivable that GLP-1 is a key player in insulin receptor regulation in the diabetic state. GLP-1 also induces its insulinomimetic effects in the normal state thereby resulting in a slight decrease of insulin receptor density in NE. DPP-IV inhibitor induces similar effects as exendin-4 on IR-α and IR-β levels and this could be attributed as well to the insulinomimetic effects of GLP-1 (Fig. 2 and 3). There is a similar attenuation in receptor density in non-diabetic DPP-IV inhibitor treated rats (N-Dp). This reflects the actions of DPP-IV inhibitor treatment on the modulation of insulin receptor density not only in the diabetic but also in the normal state.

7. Conclusion

In conclusion, insulin, Losartan, exendin-4 and DPP-IV inhibitor have demonstrated their ability as promising candidates for treating type 1 diabetic patients. Moreover, exendin-4 and DPP-IV inhibitor appear to be promising in their efficacy and prolonged antidiabetic properties. Their actions on cardiovascular function, in both the preclinical and clinical realms, warrant future investigation.

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11

Diabetic Nephrophaty in Children

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1. Introduction

Diabetic nephropathy (DN) is one of the consequences of a long-term diabetes, typically defined by macroalbuminuria—that is, a urinary albumin excretion of more than 300 mg in a 24-hour collection—or macroalbuminuria and abnormal renal function as represented by an abnormality in serum creatinine, calculated creatinine clearance, or glomerular filtration rate (GFR). [1]

2. Prevalence

Long-term microvascular and neurologic complications cause major morbidity and mortality in patients with insulin-dependent diabetes mellitus (IDDM) [2]. The incidence of overt nephropathy rapidly grows 10-15 years after the onset of type 1 diabetes mellitus; the incidence of nephropathy declines after that period and the occurence of nephropathy after 35 years of duration of type 1 diabetes is uncommon. Diabetic nephropathy rarely develops before 10 years' duration of IDDM. The peak incidence (3% per year) is usually found in persons who have had diabetes for 10-20 years. The increased mortality risk in long-term T1DM may be due to nephropathy, which may account for about 50% of deaths.
Epidemiologic data derived from one of the studies – EDC – showed that in group of patients aged <18 and with the duration of diabetes over 5 years the prevalence of microalbuminia reached as much as 14%. Long-term diabetes further increased the prevalence of albuminiria, thus 80% of male and 50% of female patients having diabetes up to 30 years of duration had proteinuria in micro- or macroalbuminuric range. ([3]-[4])

3. What is microalbuminuria

Microalbuminuria is a marker for more serious proteinuria followed by azotemia in type 1 diabetic nephropathy[5] . Microalbuminuria develops in 40-60% type 1 diabetic patients and over 50% of patients with microalbuminuria evolve to macroalbuminuric stage e.g. advanced phase of overt diabetic nephropathy.

4. Risk factor for diabetes nephropathy

Reported risk factors for the development of diabetic renal disease include a longer duration of IDDM, an earlier age at the time of diagnosis, onset of puberty, poorer glycemic control

during the first five years of diabetes, smoking, and a family history of diabetic nephropathy.[6-7]

Some of the factors leading to occurrence of diabetic nephropathy are hereditary predisposition (ACE genotype), poor glycemic control, diabetes - induced hyperfiltration, tissue hypoxia owing to reduction in capillary permeability, and increased postcapillary resistance . The capillary changes are caused by accumulation of glycated proteins and interaction of these proteins with cellular elements in the capillary walls, that effectuate the decrement in capillary ability for dilatation and ultimately, lead to capillary obstruction. In diabetes, morphological changes in type 4 collagen, heparin – sulphat proteoglycane, fibronectin and enectin molecules leads to structural and functional changes in basal membrane of the renal capillary bed .[8]

The theory of a reduction in nephron number at birth indicates that individuals born with a reduced number of glomeruli may be predisposed to subsequent renal injury and progressive nephropathy. This has been shown in animal studies in which the mother was exposed to hyperglycemia at the time of pregnancy. If this linkage is true in humans, that would have important implications concerning the role of maternal factors in the eventual development of kidney disease. [1]

Certain ethnic groups, particularly African Americans, persons of Hispanic origin, and American Indians, may be particularly disposed to renal disease as a complication of diabetes.

5. Pathology and patohistologic changes in diabetic nephropathy

The earliest morphologic abnormality in diabetic nephropathy is the thickening of the glomerular basement membrane (GBM) and expansion of the mesangium due to accumulation of extracellular matrix. Light microscopy findings in diffuse diabetic nephropathy show an increase in the solid spaces of the tuft, most frequently observed (by the positive periodic-acid Schiff reaction) as coarse branching of solid material. Large acellular accumulations also may be observed within these areas. These are circular on section and are known as the Kimmelstiel-Wilson lesions/nodules. Three major histologic changes occur in the glomeruli of persons with diabetic nephropathy. First, mesangial expansion which may be directly induced by hyperglycemia, perhaps via increased matrix production or glycation of matrix proteins. Second, a glomerulal basement membrane thickening may occur. Third, glomerular sclerosis being caused by intraglomerular hypertension (induced by renal vasodilatation or from ischemic injury induced by hyaline narrowing of the vessels supplying the glomeruli). These different histological patterns appear to have similar prognostic significance.

Hyperglycemia induces diverse metabolic changes which may give birth to variety of microvascular lesions. The glycation (also called non-enzymatic glycosilation) of proteins is a process induced by the incubation of soluble proteins in the solution with a high glucose concentrations. Glucation lead to three-dimensional structural and/or functional alterations of the proteins involved: thus, the glycation of the erythrocyte membrane proteins leads to its increased adherence ability; likewise, the glycation of heparin – sulphate proteoglycans leads to proliferation of mesangial cells.

High glucose level induces activation of polyol metabolic pathway resulting in increased sobritol production. Sorbitol, in turn, reduces intracellular myoinositol levels leading to increased capillary permeability and structural basal membrane changes. [9]

Hyperglycemia increases the expression of transforming growth factor-beta (TGF-beta) in the glomeruli and of matrix proteins specifically stimulated by this cytokine. TGF-beta may contribute to the cellular hypertrophy and enhanced collagen synthesis observed in persons with diabetic nephropathy. [10] High glucosae levels may also may activate protein kinase C, which further contribute to renal disease and other vascular complications of diabetes.

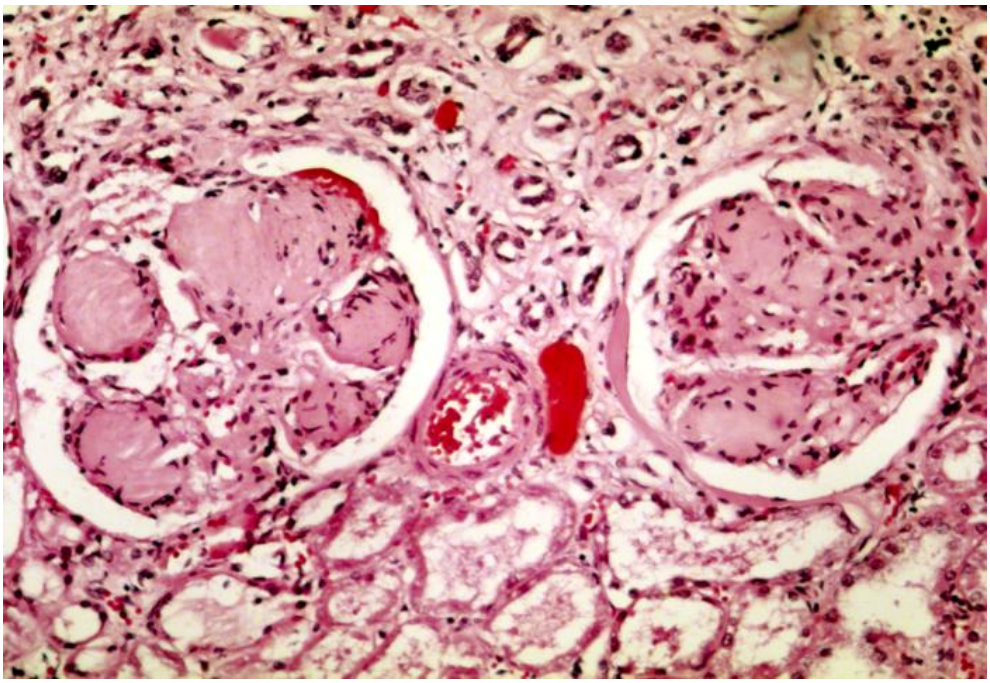


Fig. 1. Nodular glomerularsclerosis – *Nodular glomerulosclerosis in the kidney of a patient with diabetic nephropathy. US Federal Government public domain image. Source: CDC. (This image was copied from wikipediaen)*

Tissue hypoxia, augmented capillary permeability and capillary blood flow are the factors which cause increased production of angiotensin II in 45% of diabetics. Angiotensin II might generate precapillary and capillary hypertension. The presence of risk factors for hypertension is particularlly important in patients with relatively poor glucose control (hemoglobin A1 concentration above 12 percent). These patients are at increased risk of developing overt nephropathy within 20 years. [11]. Furthermore, the risk for developing diabetic nephropathy in adolescents with type 1 diabetes whose parents suffered hypertension is threefold increased. [12] The effects of prostaglandin PGI2 produced by hyperertrophic mesangial cells and atrial natriuretic polypeptide may be important in development of microvascular complications including diabetic nepropathy.

In our previous study[13], performed among the 55 pediatric patients with type 1 diabetes, who had urinary albumin excretion in the range of microalbuminuria was found in almost half (17 of 35 e.g. 48.6%) of children with diabetic ketoacidosis. Furthermore, among the 20 patients with normoalbuminuric UAE only 3 (15.0%) had ketoacidosis.

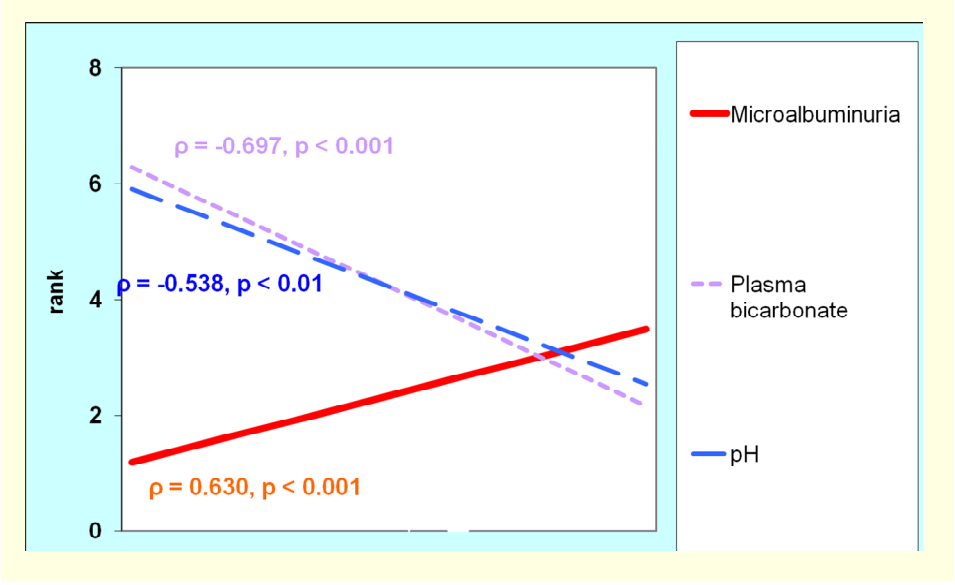


Fig. 2. Showing the correlations between the level of UAE and main parameters of acid-base staus.

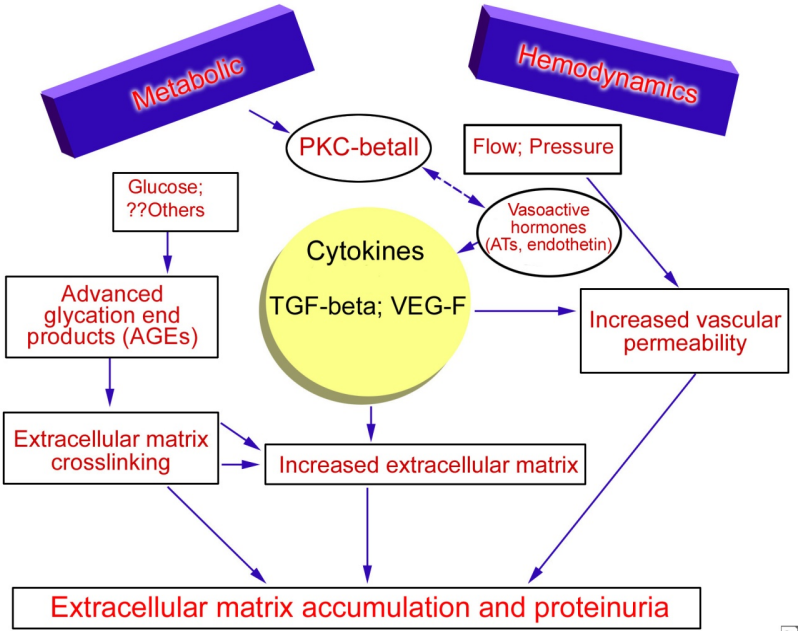


Fig. 3. The pathogenesis of diabetic nephropathy, Soman SS, Soman AS>Diabetic Nephropathy eMedicine Specialities, Endocrinology, Diabetes Mellitus, 2009.

The results of the study[13] revealed a quite significant correlation between microalbuminuria and plasma pH and bicarbonates; moreover, the level of plasma bicarbonate was a very good predictor of UAE values, depicting the aggravation of urinary albumin excretion in a response to the development of diabetic ketoacidosis

6. Alterations in renal function in diabetic nephropathy

There are 5 clinical stages of diabetic nephropathy. In the first stage, there is the substantial increase of glomerular filtration rate. Hyperglycemia and the lack of insulin along with several other hormones and factors undoubtedly contribute to development of hyperfiltration. The increase in glomerular filtration rate correlates with the enlargement of total filtration surface owing to glomerular hypertrophy; the latter reaches as much as 80% of the values existing prior to diabetes.

In the second ("silent") phase hypefunctional periods alternate the periods of normal function, and vice-versa. The periodicity of these alterations depend on quality of metabolic control. Glomerular filtration rate is, therefore, variable, arterial blood pressure is mainly within the normal range, although a mild hypertension might be present; UAE excretion rate is normal.

The third stage (the incipient nephropathy) is characterized by UAE within microalbuminuric range (30-3oo mg/24 h), there is progressive increment of arterial blood pressure, usually 5-15mmHg above the normal values, while the glomerular filtration rate may be normal, increased or reduced.

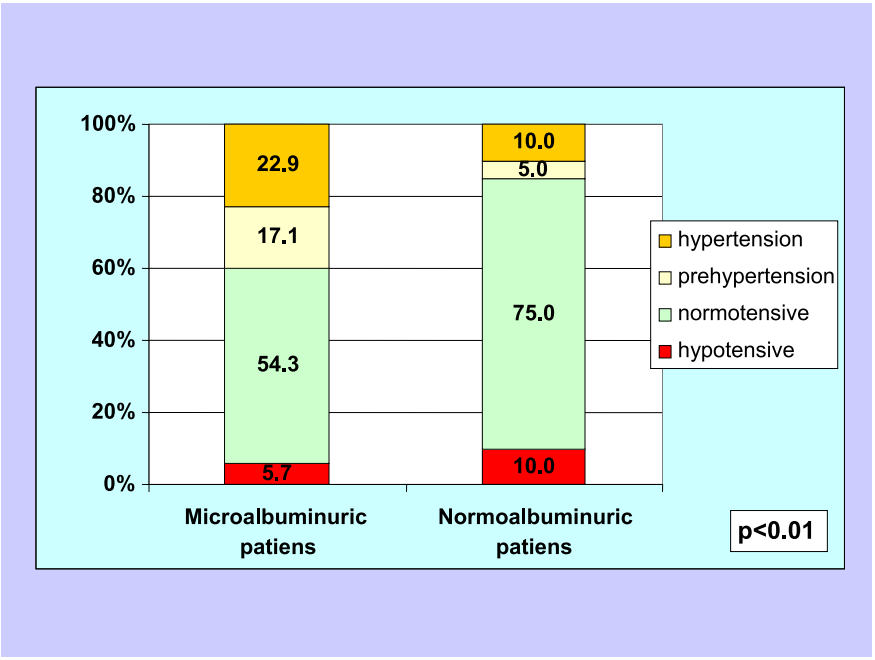
In our previous study, the total of 40% microalbuminuric children had systolic prehypertension and systolic hypertension (17.1% and 22.9%, respectively) and even 60% had diastolic blood pressure disorders: diastolic prehypertension was found in 35% and diastolic hypertension in 25% patients with microalbuminuria. While the microalbuminuric patients had significantly higher blood pressure comparing to normoalbuminuric group, its noteworthy that the percentage (15% with systolic and 20% with diastolic disturbances) of prehypertensive and hypertensive patients among the type 1 diabetic children with normal UAE was also relatively high (Fig.1 and 2). The level of blood pressure correlated significantly with UAE, but was not proved to be a predictor of microalbuminuria in children with type 1 diabetes.

The fourth stage, also known as manifest nephropathy, a clinically manifest proteinuria ensues, with UAE excretion rate exceeding 3oo mg/24h; albuminuria tends to be progressive, possibly leading to the clinical manifestations of nephritic syndrome. As nephropathy evolves to early overt stage with proteinuria (UAE >300 mg/24 hr, or >200 µg/min), it is accompanied by hypertension. The arterial blood pressure is generally raised by 7% per year, followed by the variable reduction in glomerular filtration rate. In the majority of patients, in the midst of this phase a manifest renal insufficiency characterized by overt azotemia occurs. The fourth and fifth stages almost never occur in children and are quite rare in adolescents, mostly being related to the adult population.

The fifth phase (renal insufficiency) is characterized by the general glomerular collapse, followed by overt azotemia, manifest proteinuria and grave hypertension.

Microalbuminuria is also a well-established marker of increased CVD risk [15-16]

At the beginning, proteinuria is mild and intermittent and may remain that way during 5-10 years following the first discovery. However, an increase of the amount of excreted protein and of frequency of proteinuric episodes might be expected afterwards. The beginning of retinopathy may precede or follow the occurrence of nephropathy, but in the later stages these two diabetic complications usually have roughly parallel course.



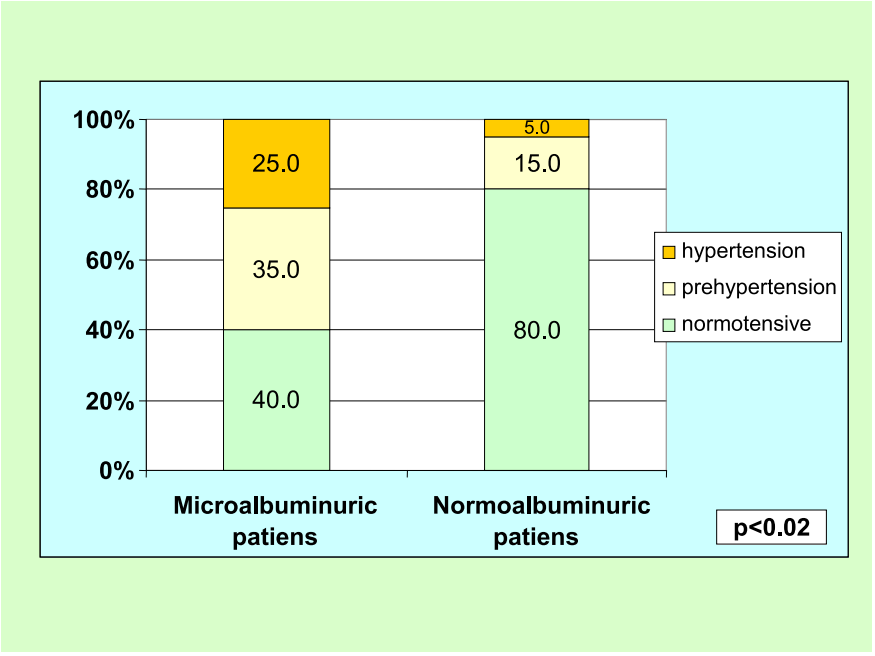


Fig. 4 and 5. Showing the percentage of blood pressure disturbances in microalbuminuric and normoalbuminuric group of children with type 1 diabetes .

Phase of constant and massive proteinuria may follow the stage of intermittent proteinuria. During the period, serum creatinine remains normal or slightly increased. The duration of this stage may be variable. Its course may accelerate leading to terminal renal insufficiency. The first sign of this acceleration is the rise in serum creatinine level.
Advanced stage nephropathy is defined by a progressive decline in renal function (declining glomerular filtration rate and elevation of serum blood urea and creatinine), progressive proteinuria, and hypertension. Progression to end-stage renal disease (ESRD) is recognized by the appearance of uremia, the nephritic syndrome.

Natural History of Diabetic Nephropathy

	Designation	Characteristics	GFR (minimum)	Albumin Excretion	Blood Pressure	Chronology
Stage 1	Hyperfunction and hypertrophy	Glomerular hyperfiltration	Increased in type 1 and type 2	May Be Increased	Type 1 normal Type 2 normal hypertension	Present at time of diagnosis
Stage 2	Silent stage	Thickend BM Expanded mesangium	Normal	Type 1 normal Type 2 may be <30-300 mg/d	Type 1 normal Type 2 normal hypertension	First 5 years
Stage 3	Incipient stage	Microalbuminuria	GFR begins to fall	30-300 mg/d	Type 1 increased Type 2 normal hypertension	6-15 years
Stage 4	Overt diabetic nephropathy	Macroalbuminuria	GFR below N	>380 mg/d	Hypertension	15-25 years
Stage 5	Uremic	ESRD	0-10	Decreasing	Hypertension	25-30 years

Fig. 6. Soman SS, Soman AS: Diabetic Nephropathy eMedicine Specialities, Endocrinology, Diabetes Mellitus, 2009.

7. The clinical features and laboratory findings in diabetic nephropathy

The first system manifestation of diabetic nephropathy is the occurrence of **peripheral oedema** usually on ankles. Contrary to the widely accepted conviction that only hypoalbuminemia gives rise to ankle oedema, the direct cause of this symptom is usually not found. It seems that the pathogenesis of this symptom is quite complex. In most of the patients, the frail capillary walls are detected very often and, thus, the oedema might be due to increased capillary permeability.
Regardless of cause, the peripheral oedema always indicate advanced stage of diabetic nephropathy and the occurrence and extent of the swelling is dependent on the duration of clinically manifest proteinuria. Also, the complaints related to lower leg muscle cramps are not unusual.
Hypertension: Incipient to mild structural glomerular lesions do not correlate with the raise in arterial blood pressure. However, in the advanced stage of diabetic nephropathy the arterial hypertension is almost always present and relates with the duration of clinically manifest proteinuria.
Some of our previous results[13] showed a very good correlation between the level of urinary albumin excretion and the values of systolic and diastolic arterial blood pressure. Still, the

arterial blood pressure level was not predictive for microalbuminuria, judging by the multiple regression analysis, as already stated above.

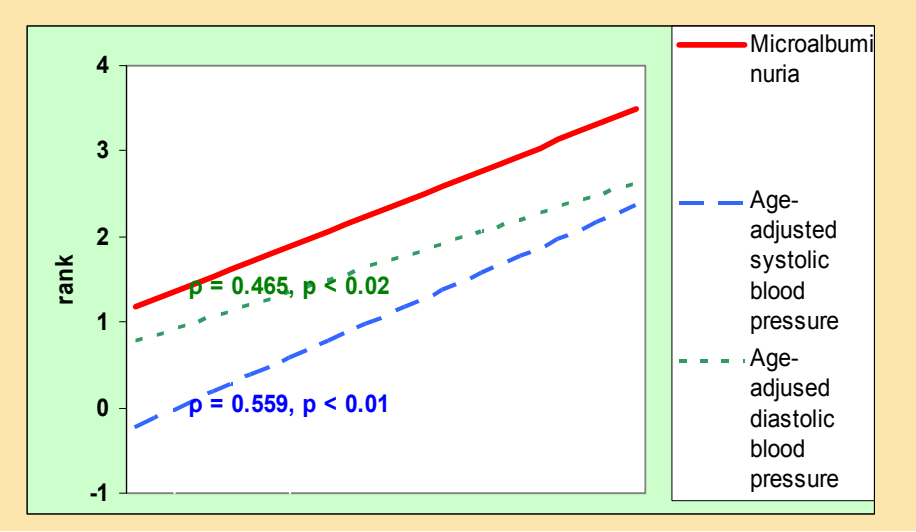


Fig. 7. Showing the relation between the arterial blood pressure level and UAE values

Paradoxally, however, there is no good correlation between the reduction in glomerular filtration rate and the rise in arterial blood pressure [17-18].Yet, most certainly, the therapy aimed at lowering high blood pressure leads to slowing down in reduction of glomerular filtration.

Macroproteinuria: The amount of proteins daily excreted in patients with diabetic nephropathy ranges from the minimal concentrations of 30 mg/day to more than 20g/day. The average excretion is about 2,5g/24h. In about 20% of patients with nephropathy the amount is less than 1g/day and in 15% it exceeds 5g/day (nephritic syndrome). Therefore, although proteinuria is considered a marker of of chronic diabetic nephropathy, yet it relatively rarely reach the nephritic range.

Other symptoms of kidney disease include loss of sleep, poor appetite, upset stomach, weakness, and difficulty concentrating. Characteristic signs are:

- Going to the bathroom more often at night
- Less need for insulin or antidiabetic medications
- Morning sickness, nausea, and vomiting
- Weakness, paleness, and anemia
- Itching

8. Differential diagnosis

Other pathological states of potential significance in differential diagnosis are:

- Cholesterol embolization
- Chronic obstruction
- Interstitial nephritis
- Amyloidosis

Disease	Differentiating Signs/Symptoms	Differentiating Tests
Non diabetic kidney disease	• Since both diabetes mellitus and chronic kidney disease (CKD) are common disorders, patients with both conditions may or may not have DN. A diagnosis other than DN should be considered if: there is a rapid progression of renal failure, evidence of another systemic disease, or short duration of diabetes (although onset is insidious in type 2, and DN may occasionally be the presenting manifestation of type 2 DM).	• Minimal proteinuria may indicate nondiabetic kidney disease. • Other specific diagnostic tests for other systemic disorders associated with nondiabetic kidney disease may be positive.
Multiple myeloma (MM)	• Multiple myeloma (MM) patients also may present with renal failure and proteinuria. • Symptoms of bone pain and anemia are the most common presenting features, affecting 80% of patients with MM.	• The characteristic test results that differ from DN are: the presence of paraproteinemia/paraproteinuria; hypercalcemia; impaired production of normal immunoglobulin; and lytic bone lesions. Bataille R, Harousseau JL. Multiple myeloma. N Engl J Med. 1997;336:1657-1664 • Urinalysis with sulfosalicylic acid (SSA) was classically utilized to evaluate for discrepancy between albumin and total protein, as standard urinalysis dipstick detects albumin only. SSA causes precipitation of all of the urinary proteins, including paraproteins (Bence Jones proteins). • Serum protein electrophoresis (SPEP), urine protein electrophoresis (UPEP): paraprotein spike. • Serum and urine free light chains: increased concentrations of free light chain in serum. • Skull x-rays, CT or MRI bone: lytic lesions. • Bone marrow biopsy: plasma cell Proliferation
Renal tract obstruction	• Can be caused by stones, cancer, fibrosis, prostate hypertrophy/cancer, neurogenic bladder, or pelviureteric junction obstruction. • Obstruction to urine flow can result in postrenal failure. Symptoms	• Passage of Foley catheter will result in flow of urine and relief of obstruction. • Kidney ultrasound: hydronephrosis, stones. • Prostate ultrasound: hypertrophy, cancer.

Disease	Differentiating Signs/Symptoms	Differentiating Tests
	include trouble passing urine, anuria, oliguria, hematuria, pain (with kidney stones), and urinary leakage/incontinence. Physical examination findings include enlarged prostate on rectal examination, costovertebral angle tenderness, suprapubic tenderness, and bladder fullness.	• CT abdomen: hydronephrosis, stones, mass, congenital abnormalities, fibrosis. • PSA: elevated in BPH, prostate cancer. • MRI: not routine but may show hydronephrosis, stones, mass, congenital abnormalities, fibrosis
Glomerulonephritis	Glomerulonephritis, such as lupus nephritis and cryoglobulinemia, is in the differential for DN. Patient presentation and physical examination may be similar to that of DN. However, there may be symptoms and signs of other systemic disease, such as rashes or joint involvement.	Urinalysis: hematuria, proteinuria, RBC casts, dysmorphic red cells. Albuminuria. Positive serology (e.g., ANA, ANCA, hepatitis serology). Complement: decreased in immune glomerulonephritis (e.g., lupus). Kidney biopsy: glomerulonephritis.
Renal artery stenosis	Renal artery stenosis presents either as hypertension refractory to multiple maximized antihypertensives or as renal failure shortly after the initiation of an ACE inhibitor. Physical examination is significant for an abdominal bruit. Safian RD, Textor SC. Renal-artery stenosis. N Engl J Med. 2001;344:431-442	Ultrasound, CT scan, MRI: shrunken kidney, decreased flow through the renal artery. Magnetic resonance angiography (MRA): renal artery stenosis. Renal angiogram: renal artery stenosis.

Table 1. BMJ Group: Diabetic nephropathy, diferential diagnosis , epocrates online, 2010

8.1 The diagnosis of diabetic nephropathy [19]

Patients with diabetes should be screened annually for DKD. Initial screening should commence:

- 5 years after the diagnosis of type 1 diabetes; or
- From diagnosis of type 2 diabetes.

Screening should include:

- Measurements of urinary ACR in a spot urine sample;
- Measurement of serum creatinine and estimation of GFR.

An elevated ACR should be confirmed in the absence of urinary tract infection with 2 additional first-void specimens collected during the next 3 to 6 months.

- Microalbuminuria is defined as an ACR between 30-300 mg/g.
- Macroalbuminuria is defined as an ACR > 300 mg/g.
- 2 of 3 samples should fall within the microalbuminuric or macroalbuminuric range to confirm classification.

Using the CKD staging likelihood of DN can be determined as follows:

- Normoalbuminuria in CKD stages 3 to 5 (GFR <60) is unlikely to be DN.
- Microalbuminuria in CKD stages 1 to 3 (GFR >30) is possible DN.
- Microalbuminuria in CKD stages 4 to 5 (GFR <30) is unlikely to be DN.
- Macroalbuminuria at all stages of CKD is highly likely to be DN.

Urinanalysis	proteinuria
Urinary albumin for creatinine ratio	microalbuminuria: between 30 and 300 mg/g; macroalbuminuria: >300 mg/g
Blood biochemistry	elevated creatinine
Serum creatinine with GFR estimation	Glomerular filtration rate (GFR) may be raised in CKD stage 1, normal in CKD stage 2, and reduced in CKD stages 3 to 5
Kidney ultrasound	normal-to-large kidneys with increased echogenicity; may show hydronephrosis if vesiculopathy and/or obstruction is superimposed

Table 2. Clinical tests in diagnosis of diabetic nephropathyClinical tests in diagnostics of diabetic nephropathy:

Test	Results
24 hour urine to collection	microalbuminuria: albumin 30 to 300 mg/24 hours; macroalbuminuria: albumin >300 mg/24 hours
CT-abdomen	hydronephrosis; wedge-shaped areas of low attenuation; loss of the ability to distinguish the corticomedullary border; perinephric stranding; cysts; masses; stones
Magnet resonance angiography	renal artery stenosis
Doppler ultrasound	may show renal artery stenosis
Kidney biopsy	mesangial expansion, fibrosis, Kimmelstiel-Wilson nodules

The source: epocrates online com, 2010

Table 3. Other tests to consider

High blood pressure often goes along with diabetic nephropathy. You may have high blood pressure that develops rapidly or is difficult to control.

9. Treatment and therapy

Annual screening for microalbuminuria, with a random spot urine sample for microalbumin-to-creatinine ratio, should be initiated once the child is 10 years of age and has had diabetes for 5 years.

Confirmed, persistently elevated microalbumin levels on two additional urine specimens should be treated with an ACE inhibitor titrated to normalization of microalbumin excretion if possible

Once albuminuria is diagnosed, a number of factors attenuate the effect of hyperfiltration on kidneys:

1. Control of hyperglycemia- The Diabetes Control and Complications Trial (DCCT) [21] and the United Kingdom Prospective Diabetes Study (UKPDS) [21] have definitively shown that intensive diabetes therapy can significantly reduce the risk of the development of microalbuminuria and overt nephropathy in people with diabetes.

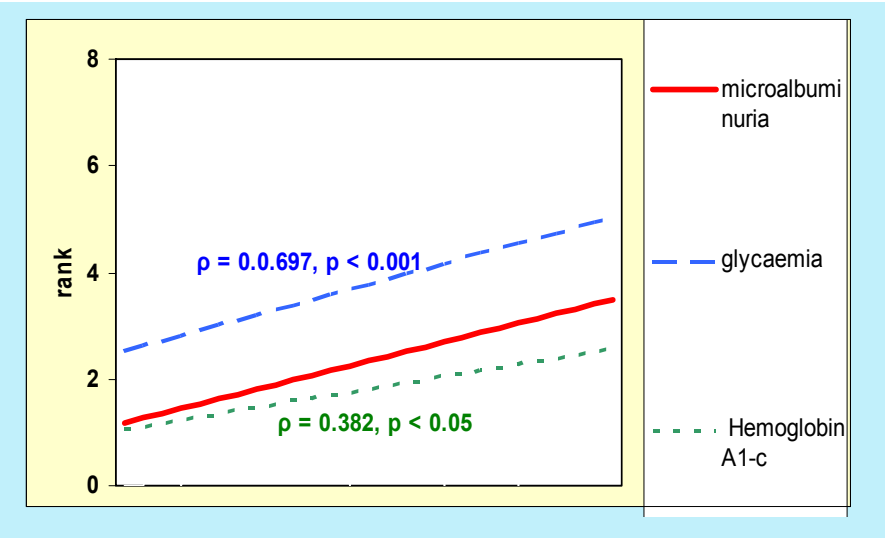


Fig. 8. Showing the relation of the parameters of metabolic regulation and the UAE level in children with type 1 diabetes

Accordingly the earlier study[13] revealed that hemoglobin A1/c and, particularly, blood glucose level were significantly related to urinary albumin excretion rate. Furthermore, blood glucose was the major predictor of microalbuminuria in children with type 1 diabetes. Lowering A1C to an average of ~7% has been shown to reduce microvascular and neuropathic complications of diabetes and, possibly, macrovascular disease. Preliminary results of the multicenter A1C-Derived Average Glucose (ADAG) Trial, presented at the European Association for the Study of Diabetes meeting in September 2007, confirmed a close correlation of A1C with mean glucose in patients with type 1, type 2, or no diabetes. Final results of this study, not available at the time this statement was completed, should allow more accurate reporting of the estimated average glucose (eAG) and improve patients' understanding of this measure of glycemia[22]

Target HbA1c for people with diabetes should be < 7.0%, irrespective of the presence or absence of CKD. [23]

Considering the high incidence of overnight hypoglycemias, in children and adolescents during the optimal regulation of blood glucosae levels, the A1C level achieved in the "intensive" adolescent cohort of the DCCT group was >1% higher than that achieved by adult DCCT subjects and above current ADA recommendations for patients in general. Standards of Medical Care in Diabetes—2008 American Diabetes Association Tight glycemic control will delay the progression of microalbuminuria and slow the progression of diabetic nephropathy.

2. Aggressive control of systemic blood pressure- In patients with type 1 diabetes, hypertension is usually caused by underlying diabetic nephropathy and typically becomes manifest about the time that patients develop microalbuminuria . Hypertension in childhood is defined as an average systolic or diastolic blood pressure ≥95th percentile for age, sex, and height percentile measured on at least three separate days. "High-normal" blood pressure is defined as an average systolic or diastolic blood pressure ≥90th but <95th percentile for age, sex, and height percentile measured on at least three separate days.

Treatment of hi gh-normal blood pressure (systolic or diastolic blood pressure consistently above the 90th percentile for age, sex, and height) should include dietary intervention and exercise aimed at weight control and increased physical activity, if appropriate. If target blood pressure is not reached with 3–6 months of lifestyle intervention, pharmacologic treatment should be initiated.

Pharmacologic treatment of hypertension (systolic or diastolic blood pressure consistently above the 95th percentile for age, sex, and height or consistently >130/80 mmHg, if 95% exceeds that value) should be initiated as soon as the diagnosis is confirmed.

The principal agents for lowering high blood pressure are angiotensin convertase inhibitors (ACE). The beneficial effect of ACE inhibition on preventing progression from microalbuminuria to overt diabetic nephropathy is long-lasting (8 y) and is associated with the preservation of a normal GFR. ACE-I reduces the risk of progression of overt type 1 diabetic nephropathy to ESRD and in type 1 patients with microalbuminuria to overt nephropathy. [25] The results of multicentric studies showed that blood pressure in patients with chronic renal insufficiency should be lowered below normal range (as recommended by WHO) in order to achieve beneficial effect on progression of the disease.

A meta-analysis of several small studies has shown that protein restriction may be of benefit in some patients whose nephropathy seems to be progressing despite optimal glucose and blood pressure control [26]

Hypertensive people with diabetes and CKD stages 1-4 should be treated with an ACE inhibitor or an ARB (Angiotensin Receptor Blocer), usually in combination with a diuretic[27]

If needed to achieve blood pressure targets, a thiazide diuretic should be added to those with an estimated glomerular filtration rate (GFR) (see below) ≥50 ml/min per 1.73 m2 and a loop diuretic for those with an estimated GFR <50 ml/min per 1.73 m2. (E)

Normotensive people with diabetes and macroalbuminuria should be treated with an ACE inhibitor or an ARB.In type 1 diabetes with macroalbuminuria, ACE inhibitors decrease albuminuria and reduce the risk of clinical outcomes regardless of the presence or absence of hypertension. A randomized controlled trial in people with type 1 diabetes and macroalbuminuria found that ACE inhibitors reduced the risk of the combined outcome of doubling of serum creatinine level, CKD stage 5, and death. [28] A quarter of the participants were normotensive. There was no significant difference in the treatment effect between the normotensive and hypertensive individuals.

Future agents: Wenzel et al. [29] . have examined the role of avosentan (endothelin antagonist) on progression of microalbuminuria. Avosentan have demonstrated antifibrotic, anti-inflammatory, and antiproteinuric effects in experimental studies. Wenzel et al conducted a randomized, placebo-controlled, double-blind, parallel-design, dosage-range study on the effect of the endothelin-A antagonist avosentan (SPP301) on urinary albumin excretion rate in 286 patients with diabetic nephropathy, macroalbuminuria, and a blood pressure of <180/110 mm Hg. All dosages of avosentan, administered in addition to standard ACE inhibitor/ARB treatment, were found to reduce the mean relative urinary albumin excretion rate (-16.3% to -29.9%, relative to baseline) in the study's patients.

3. Selective control of arteriolar dilation by use of angiotensin-converting enzyme (ACE) inhibitors (thus decreasing transglomerular capillary pressure) This is particularly significant when lowering of systemic blood pressure is accompanied with concomitant lessening of glomerular capillary pressure. however the optimal lower limit for systolic blood pressure is unclear. [30]

4. Dietary protein restriction (because high protein intake increases renal perfusion rate).

A meta-analysis examining the effects of dietary protein restriction (0.5-0.85 g/kg/d) in diabetic patients suggested a beneficial effect on the GFR, creatinine clearance, and albuminuria. However, a large, long-term prospective study is needed to establish the safety, efficacy, and compliance with protein restriction in diabetic patients with nephropathy. Considering the importance and role of dietary proteins in the process of growth and development, a sharp restriction (<15% of total daily amount of nutrients) of proteins in adolescents with diabetic nephropathy is not justified.

Other standard modalities for the treatment of progressive renal disease and its complications (e.g., osteodystrophy) must also be used when indicated, such as sodium and phosphate restriction and use of phosphate binders. When the GFR begins to decline substantially, referral to a physician experienced in the care of such patients is indicated. Radiocontrast media are particularly nephrotoxic in patients with diabetic nephropathy, and azotemic patients should be carefully hydrated before receiving any procedures requiring contrast that cannot be avoided. [31]

As for any other patient with ESRD, diabetic patients with ESRD can be offered hemodialysis, peritoneal dialysis, kidney transplantation, or combined kidney-pancreas transplantation.

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12

Understanding Pancreatic Secretion in Type 1 Diabetes

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Brazil

1. Introduction

Type 1 Diabetes Mellitus (T1DM) is a chronic disease characterized by the immune-mediated destruction of β cells of pancreatic islets. Despite the increase of its incidence observed in last decades, it has not been fully elucidated the immunogenetic and environmental factors associated with the initiation and perpetuation of the pancreatic injury(1,2).

The pathogenic process in T1DM begins with insulitis, which progresses and expands so as to be accompanied by cell necrosis. The rate of progression of the lesion is variable and may be related to age at onset of the disease, being faster in cases diagnosed in children (3). When approximately 80-90% of insulin-secreting cells have been destroyed, T1DM is clinically overt (4).

T1DM patients may have some residual insulin secretion at diagnosis (5-8). Several studies indicate that the loss of functional capacity usually occurs within three to five years. Most patients with long duration of disease do not present clinical evidence of preservation of β cells (9-11). However, the conservation of some residual secretion, even insufficient to cure or prevent T1DM from using insulin, has been associated with a better prognosis regarding glycemic control, lower rates of hypoglycemia, diabetic retinopathy and nephropathy (12).

One of the most frequently used method to assess β cell function in patients with T1DM is the determination of C-peptide (CP), a molecule secreted by β cells in equimolar concentrations with insulin and without significant liver metabolism (13-15). Taking into consideration the potential benefits of preserving a residual insulin secretion in patients with T1DM, experimental treatments have been proposed in order to maintain detectable levels of CP in these individuals.

Developments in research aiming for the prevention and cure of T1DM, increased the need to elucidate the contributing factors for the maintenance of residual insulin secretion in patients affected by the disease. This is because individuals who still exhibit residual insulin secretion could be ideal candidates for new curative treatments. Furthermore, most studies of pancreatic function in T1DM have been performed in Caucasians and Asians. We do not know whether the results obtained so far can be extrapolated to other ethnic populations (16).

2. Type 1 diabetes mellitus (T1DM)

2.1 Definition

T1DM is a chronic disease characterized by the destruction of pancreatic β cells, which leads to absolute deficiency in insulin production.
There are two subgroups of T1DM: type 1A, mediated by autoimmune destruction of β cells and type 1B, without an identifiable cause and more common among Asian and Afro-American people (17).

2.2 Epidemiology

The incidence of T1DM is rising in recent decades (18-20). This has been observed mainly in developed countries, mainly among children younger than four years-old (18,20-22). It is estimated that 15 to 30 million people in the world population have the disease with a growth rate of approximately 3-5% per year (19,20,23,24).
Epidemiological data suggest that 30 to 50% of T1DM cases may occur after the age of 20 and 50-60% of these patients were younger than 16-18 years at diagnosis. The incidence declines throughout the adult life (25-28).
The incidence among men and women is equal. However, there is a predominance of females in populations at low risk for T1DM, while the opposite occurs in high-risk populations (29).
There is also variation in relation to different countries. There is a high incidence in Finland and Sardinia, Italy (36.5 and 36.8 individuals per 100,000, respectively) and low in Germany and Pakistan. In countries like Brazil and Portugal, the incidence of T1DM is intermediate, as shown in Table 1. In Brazil, there is about 8.0 new cases per 100,000 inhabitants (30).
Regarding ethnicity, individuals of Caucasian origin have a higher incidence of T1DM than Hispanic, African, Asian or Indian descendents (31).

	Incidence per 100,000 people	
Country	**Term**	**Total**
Germany	1990 – 1994	1.0
Brazil	1990 – 1992	8.0
Canada	1990 – 1994	24.0
Denmark	1990 – 1994	15.5
Spain	1990 – 1994	12.5
USA	1990 – 1994	
White		16.4
Non-white		13.3
Finland	1987 – 1989	36.5
Italy	1990 – 1994	
Sardinia		36.8
Sicily		11.7
Lombardy		7.2
Mexico	1990 – 1993	1.5
Norway	1990 – 1994	21.2
Pakistan	1990	0.7
Portugal	1990 – 1994	13.2
Sweden	1978 – 1987	24.2

Adapted from The WHO Diamond Project Group, 2000 (30).

Table 1. Incidence of T1DM in population under 15 years-old, in different countries.

2.3 Etiopathogenesis

In the pathogenesis of T1DM, the activation of the immune system mediated by T cells plays a central role. This process leads to an inflammatory reaction (insulitis) characterized by infiltration of pancreatic islets by mononuclear cells such as dendritic cells, macrophages, B lymphocytes and CD4 and CD8 (3,17,32). With the progression of inflammatory injury, there is a development of cellular necrosis and cellular immunity seems to be primarily responsible for this process (33).

The pathogenic process that culminates with the onset of T1DM begins with the loss of self-tolerance of T lymphocytes. Self-tolerance is defined as the process in which T cells or autoreactive B are eliminated from the body. This can be divided into central and peripheral. The central tolerance is the deletion of autoreactive T cells in the thymus and requires the presence of autoantigens in thymic environment. Peripheral tolerance mechanisms are responsible for destroying or inhibiting the function of self-reactive cells that crossed the thymic deletion process, through mechanisms such as immunological ignorance, deletion, anergy or immune inhibition (34).

Some of the risk genes for the development of T1DM are responsible for the concentration of insulin within the thymus. Thus, these genes are linked to the process of central tolerance. Moreover, changes in peripheral tolerance may also be related to the etiopathogenesis and the release of super-antigens after viral infections. Typically, the peripheral tolerance would be responsible for the sequestration of these super-antigens mediated by the immune system, which would not lead to lymphocyte activation (34,35). Another mechanism of pancreatic injury is associated with infection as a possible molecular mimicry between viral antigens and autoantigens aggravating the process of insulitis. This molecular similarity could activate autoreactive T lymphocytes and direct them to attack pancreatic cells (34).

Overt T1DM occurs when approximately 80 to 90% of the β cells have been destroyed (4). The rate of progression to classical T1DM symptoms may be related to age of onset of symptoms, being faster in cases diagnosed at young age (3).

The presence of one or more autoantibodies associated with T1DM may precede the onset of clinical disease by months or even years (28,36,37).

T1DM diagnosed in childhood may have autoantibodies to the major antigens of the pancreatic islets detectable in the first two years of life (38). This seems to be a reflection of cellular injury and its cause is not specific (33). The role of B lymphocyte cells in the pathogenesis of T1DM must also be emphasized. Several diseases mediated by T lymphocytes present B lymphocytes in the process of antigen presentation (39). A recent study revealed that patients with newly diagnosed T1DM treated with Rituximab (monoclonal anti-CD20) showed improvement in clinical and metabolic parameters after selective depletion of B lymphocytes. The explanation for this fact lies on the reduction of antigen presentation mediated by B lymphocytes, or even the reduction of cytokine production in pancreatic or peri-pancreatic lymph nodes. This suggests that B lymphocytes may have a greater role in the pathogenesis of T1DM than previously thought (40).

Although there are many hypotheses about the onset and progression to autoimmunity of T1DM, the definitive mechanism is not fully understood.

2.4 Autoantibodies

Production of pancreatic autoantibodies does not appear to be the primary mechanism of destruction of pancreatic islets (41,42). The release of antigen caused by β cell destruction leads to its detection by the immune system and immune activation, subsequently causing

the production of antibodies against pancreatic components. Thus, antibodies appear to be predominantly markers of immune activation and β cell destruction, and not the cause itself (31,33).

The first autoantibody isolated was the antibody against the cytoplasm of the pancreatic islet *(islet cell cytoplasmic antibody* - ICA*)* and three major autoantibodies contribute to their positivity: the antiglutamic acid decarboxylase (GADA65), antibody against tyrosine phosphatase (anti-IA2, also known as ICA512) and antibodies against glycolipids (43)

The glutamic acid decarboxylase (GADA) is an enzyme involved in the synthesis of γ-amino-butiric acid (GABA) in the central nervous system (CNS) and in pancreatic islets. GADA is expressed in all cell types of pancreatic islets, not only in β cells. Two isoforms of GADA (GADA64 and GADA67) synthesized in other tissues have also been identified. GADA, used in current assays, detects pancreatic isoform of this enzyme of 65 kd and is found in 70-80% of newly diagnosed Caucasian. A lower incidence is observed in children younger than 10 years of age (43,44).

After GADA discovery, anti-IA-2 (40 kd) or ICA512, and anti-IA-2β (37 kd), known as *phorin* (45,46), were identified. Most patients with anti-IA 2β also exhibit anti-IA-2. However, about 10% of T1DM with anti-IA-2 autoantibodies are not the type-2 anti-IA. Until now, anti-IA-2 appears to be the most specific immunological marker of T1DM and is present in 32 to 75% of the new cases (47,48). Once detected, most individuals concomitantly present positive GADA and anti-insulin (IAA) (28). Another antibody has been identified as IAA. In children diagnosed under 10 years of age, sensitivity of IAA is of 50 to 60%, while in patients between 10 and 30 years sensitivity is of 10% (47,48). There is cross-reactivity between antibodies produced against endogenous and exogenous insulin and most patients develop IAA after initiation of insulin therapy, even with the use of recombinant analogues. Thus, the measurement of this antibody in the blood is recommended only before or within 5 to 7 days after initiation of insulin therapy (32).

It has been recently described another auto-antigen associated with T1DM - the zinc transporter 8 *(zinc transporter* 8 - Znt8). Present in β cells, the Znt8 is involved in regulating the insulin secretion pathway. It regulates the entry of zinc in the lumen of the granules, where this cation binds to hexamers of insulin. Nearly 60 to 80% of individuals with newly diagnosed T1DM have antibodies against this antigen (49,50).

Most pancreatic autoantibody titers decline after the diagnosis of the disease, but GADA may remain positive for many years after diagnosis (51). This feature makes it ideal for antibody studies in patients with T1DM of long duration.

Some patients may not have pancreatic autoantibodies detectable at diagnosis. This can be explained by some reasons (5):

1. These patients may have detectable titers in the pre clinical and became negative before diagnosis.
2. Tests available for determination of these autoantibodies were not sensitive enough to detect low titers.
3. There may be yet unidentified antibodies in research conducted so far.
4. This is possibly a case of idiopathic T1DM (type 1B) without autoimmune etiology.

At diagnosis, traditionally it has been estimated that 90% of children had one or more pancreatic antibody positive. However, with the availability of anti-Znt8, measuring the combination of the four main pancreatic autoantibodies (GADA, IAA, anti-IA2 and anti-Znt8) in patients with newly diagnosed T1DM, showed a detection rate of autoimmunity against β cells of 98% (49,50).

2.5 Genetic factors associated with T1DM

Genes from the histocompatibility system HLA *(Human Leukocyte Antigen Complex), IDDM* 1 - chromosome 6q21.31, are polymorphic and have different amino acid sequences between individuals. They are divided into HLA class I (HLA-A, B and C) and HLA class II (DP, DQ and DR) and both are related to immune response (52).

HLA class II polymorphisms are associated with an increased risk of T1DM. The main risk alleles are HLA DQB1 * 2002 / * 0302 and HLA DR03/04. The DQB1 * 0602 allele is considered protective (5).

Other genes have been associated with the pathogenesis of T1DM, as shown in Table 2, among which are the insulin gene PTPN22 and CTL4 (17,27,53-55).

* IDDM - insulin dependent diabetes mellitus	Genetic Product	Cromossomic location
IDDM1	HLA	6p21.31
IDDM2	Insulin	11p15.5
IDDM3	-	15q.26
IDDM4	-	11q13
IDDM5	-	6q25
IDDM6	-	18q21
IDDM7	-	2q31-31
IDDM8	-	6q27
IDDM9	-	3q21
IDDM10	-	10p11-q11
IDDM11	-	14q4.3-14q31
IDDM12	CTLA-4	2q33
IDDM13	-	2q34
IDDM14	-	6q21
IDDM15	-	10q25.1
IDDM17	-	CR10
PTPN22	-	

Adapted from Kelly et al. 2003 (3) and Eisenbarth, 2005 (32).

Table 2. Genes involved in the Pathogenesis of T1DM

2.6 Environmental factors associated with T1DM

Besides genetic susceptibility, exposure to environmental factors is also important for the development of T1DM. Studies suggest that these factors would be responsible for triggering the immune process that leads to β cell destruction. Specific viral infectious diseases have been included among the causes (56,57). Some of the viruses suggested as associated with T1DM are Enteroviruses, Coxsackie virus, congenital Rubella. Toxins such as nitrosamines and protein foods such as cow's milk, cereals and gluten are also considered as potential immunological triggers (58-60). Moreover, the presence of multiple infections in early life is associated with a reduced risk for the disease (1).

Recently, there has been a significant increase in diagnosed cases aged younger than four years (61,62). This shift may be explained by increased exposure to environmental factors or the increased prevalence of obesity (63,64).

One of the hypotheses to explain the development of T1DM is the controversial theory of acceleration suggested by Wilkin. It argues that T1DM and type 2 diabetes (T2DM) constitute a single disease and not two distinct comorbidities. The rate of loss of β cell mass, associated with three main factors accelerators, would define the disease. The first factor would be the intrinsic potential for high speed apoptosis of β cells, essential, but insufficient for the development of DM. The second accelerator would be insulin resistance, a result of obesity and physical inactivity, central link between the two entities. Insulin resistance overtaxes the β cell mass already at risk for accelerated apoptosis, contributing to the clinical expression of DM. The third accelerator was present only in individuals with genetic susceptibility to autoimmunity. Individuals with metabolically more active β cells, insulin resistance and genetic susceptibility would be more prone to rapid deterioration of functional and clinical expression of T1DM. In the absence of autoimmune accelerator, apoptosis rate would be slower and thus there would be progression to T2DM (65).

3. Assessment of pancreatic β cell function

A major limitation of studies of T1DM in humans is the inability to measure the mass of β cells *in vivo,* since pancreatic biopsies are associated with high morbidity and mortality. Therefore, indirect methods have been developed. The assessment of pancreatic function was shown to have a rough correlation with the mass of β cells used in islet transplantation in patients with DM (25).

Methods of imaging and nuclear medicine are being studied to assist the measurement of the mass of pancreatic islets and its correlation with insulin production, but with conflicting results (66-68).

The measurement of β cell mass does not always correlate with functional capacity. In pre-diabetes, there can be no proliferation or maintenance of cell mass (69,70). Marchetti et al suggested that patients with T1DM can have β cell secretory dysfunction and not just cell destruction (71).

In an attempt to understand the β cell function *in vivo,* it was initially developed a radioimmunoassay for measurement of serum insulin. For years, this was the gold standard for assessing the secretory activity. However, there are several factors limiting the use of serum insulin for the evaluation of pancreatic β cell function. The first is that 50 to 60% of the insulin produced by the pancreas undergoes hepatic metabolism and does not reach the systemic circulation. In addition, the peripheral *clearance* of insulin is variable and the tests available for their determination do not differentiate insulin, proinsulin, its intermediates

and the use of exogenous insulin. Another limiting factor is the presence of anti-insulin antibodies (IAA) to interfere in the measurement of serum insulin (12,72,73).
Thus, other means to measure β cell function have been developed, and among them the measurement of baseline and/or stimulated CP.

3.1 C-peptide

Pancreatic β cells secrete, in addition to insulin, proinsulin, conversion intermediates of insulin (proinsulin *split)* and the connecting peptide (CP) (15).
The pro-insulin is cleaved in the Golgi apparatus of islet cells. This reaction leads to the formation of insulin, CP and two pairs of basic amino acids. Insulin and the CP are released into circulation at a ratio of 1:1, as well as small amounts of proinsulin and intermediates. The proinsulin sum 20% of molecules with insulin-immunoreactivity *simile,* seems to have no metabolic effect, undergoes extra-hepatic metabolism and is excreted exclusively by the kidneys.
The CP is a connection between the peptide chains A and B of proinsulin and facilitates the processing of biologically active insulin in secretory granules of pancreatic islets. After the cleavage of proinsulin, the intact CP is stored with insulin in these granules and is co-secreted with insulin. For this reason, the CP can be considered an independent marker of insulin secretion (16). However, in some situations, such as renal failure, the serum concentration of CP is not proportional to the rate of insulin secretion. About 85% of CP is metabolized by the kidneys and the remainder excreted intact in urine. A decrease in renal function leads to reduced metabolism of CP and elevated serum levels (74)
The CP plasma half-life is of thirty minutes, greater than that of insulin, which is only of four minutes (13-15,75). The normal value of the CP varies from 1.1 to 5.0 ng / mL.
Under standard conditions of measurement, the CP has been widely accepted as a rough measure of insulin secretion, since it is secreted into the portal circulation in equimolar, concentrations, does not undergo hepatic metabolism, its half-life is longer (30 minutes) and has low cross reactivity with proinsulin and insulin antibodies (12, 76,77).
In adverse conditions, such as hyper- or hypoglycemia, CP concentrations are not proportional to the rate of insulin secretion, and its *clearance* may vary between different individuals (78,79).
Therefore, at the moment, the most appropriate, accepted and clinically validated method to measure the ability of β cell secretion under ideal conditions is the measurement of baseline and/or stimulated CP (76). This stimulation can be done with glucose or insulin secretagogues such as glucagon, standard mixed meal or oral glucose tolerance test with 75g of anhydrous glucose (OGTT) (12,80-82).
The standard mixed meal test consists of oral administration of a liquid diet (Sustacal ® / Boost) of approximately 500 kcal containing 50% carbohydrate, 30% fat and 20% protein. Blood samples for measurement of blood glucose and CP are collected in fasting and 30, 60 and/or 90 minutes after the meal (76,80). This test shows the typical postprandial response of the cell β and its interaction with the various hormones secreted during oral feeding. It is the most physiological test among the above cited (76,83).
Oral glucose tolerance test (OGTT) is the determination of glucose, insulin and CP after 10 hours of fasting and 30, 60, 90, 120 minutes after ingestion of 75g anhydrous glucose orally administered. It is used to measure glucose tolerance and the residual function of the β cell in patients at risk, but has not yet been validated for use in T1DM patients. It is useful to

predict early changes in glucose metabolism in relatives of T1DM or individuals with positive autoantibodies (76).

The glucagon stimulation test is done by measurement of CP at baseline, after 8 hours fasting, and 6 minutes after intravenous administration of 1 mg of glucagon, while its maximum concentration is observed (76,79). The most commonly observed side effects are facial flushing and nausea due to decreased gastrointestinal motility (76). The advantages of the glucagon test in relation to the standard meal test are:

1. Faster action, since glucagon is a potent supra-physiological stimulus for insulin secretion, directly and indirectly, also influenced by hyperglycemia (76).
2. Minor influence of glucotoxicity in patients with high glycated hemoglobin (HbA1C) (76,80).
3. Simple technical achievement (76).
4. Good reproducibility between individuals (76).

To avoid inaccuracy in CP measurement, caution is necessary during blood collection and processing. As the CP is a small molecule, linear and prone to degradation by proteolytic enzymes blood samples should be cleared by centrifugation for a short period of time (not more than a few hours). Palmer et al suggest that this is done within one month after collection because immunoreactivity falls with prolonged blood storage generating falsely lower results (84). This time, however, it is not well defined (12). Until the proper measurement, the serum should be stored -80 ° C.

Changes in blood glucose are factors that acutely interfere with the measurement of serum CP and can underestimate the ability of secretion. The Immunology of Diabetes Society has established that the glucagon test must be conducted during fasting and with glucose levels between 70 and 200mg/dL. The optimal level of blood glucose in these patients is around 126mg/dL. Hypoglycemia (<70 mg / dL) inhibits the insulin response while acute hyperglycemia (> 200mg/dL) may potentiate the secretory response or inhibit it. On the other hand, chronic hyperglycemia can reduce β cell function due to the phenomenon of glucotoxicity (85-86). Moreover, high glucose concentrations have been shown to damage β cells *in vitro* and *in vivo,* compromising insulin secretion (87).

The characteristics of the methods used to quantitate the CP must be well defined. The presence of cross-reactivity with proinsulin and its intermediate results in falsely elevated CP concentrations. In general, it is expected that the rate of cross-reactivity of a method is less than 10% (12).

3.2 Role of the pancreatic β cell in T1DM

In T1DM, there is a progressive loss of the ability of insulin secretion. During the process of destruction of pancreatic β cells, the first abnormality observed in the preclinical phase is the loss of first phase insulin secretion - *FPIR, First phase insulin release* (31,88). The FPIR is the sum of the plasma insulin at 1 and 3 minutes after the glucose load during an intravenous glucose tolerance test (76). Moreover, impairment in glucose tolerance test has been correlated with an increased risk of progressing from preclinical to clinical diabetes (4).

One of the first authors who studied β cell function in diabetic patients and healthy controls was OK Faber et al in 1977. He compared measurements of the CP after the standard meal and after glucagon (1 mg intravenous) and noticed that the CP, at baseline and after fasting and stimulation with glucagon, was higher in healthy controls when compared to patients with T1DM (79). Subsequently, it was identified that individuals with T1DM may have

some residual insulin secretion at diagnosis especially in cases diagnosed in adulthood (5-8, 89).
The loss of this residual secretion usually occurs within three to five years after the diagnosis (86). Patients with long duration of illness tend not to have CP secretory reserves, demonstrating the exhaustion of pancreatic secretion (9-11). However, Meier et al demonstrated the preservation of β cells secreting insulin in most patients with T1DM evaluated in histopathological studies (90). In addition, Keenan and colleagues demonstrated detectable levels of CP in 18% of individuals with T1DM for over 50 years and absence of chronic complications of the disease (91).
The reason why some β cells are maintained for years after diagnosis of T1DM remains unclear. It is possible that some cells are not equally susceptible to destruction or even that the destructive process is attenuated over the years. Another possibility to explain this persistence would be a recovery in β cell by replication, which seems unlikely. Another explanation is that some cells could be inactive and not destroyed and recover their function over time (92).

3.2.1 Factors influencing the residual insulin secretion and CP

The rate of decline of pancreatic function in T1DM is heterogeneous, ranging from 13 to 58% in the first year after diagnosis (84). Some factors such as age at diagnosis and sex seem to influence this fall. Association between residual pancreatic function and female sex has also been found (93). Karjalainen et al reported that T1DM that begins in adulthood (20 to 55.8 years old) is characterized by a longer asymptomatic period before diagnosis and better preservation of residual β cell function than T1DM beginning in childhood (5,16,94).
Some studies have shown that baseline serum CP levels in patients diagnosed in adulthood and post-pubertal period are higher than in those diagnosed in the pre-puberty (7,8,12). This fact indicates a greater destruction of β cells in younger people. However, there could be a change in CP levels according to age. According to Palmer JP et al, the serum CP levels in adulthood are around 0.6 to 1.3 nmol / L during puberty between 0.3 and 0.9 nmol / L and in pre-pubertal <0.2 nmol / L (12).
In the *Diabetes Control and Complications Trial* (DCCT), patients with short disease duration (≤ 5 years) had a CP after the stimulus with mixed meal detectable (greater than 0.2 nmol / L) in 33% of those with <18 years of age and 48% in adults (10). Stimulated CP > 0.2 pmol / mL was found in 3% of children and 8% of adults with long duration of illness (> 5 years). Basal and stimulated CP were negatively correlated with disease duration (80).
The presence of antibodies is another predictor of reduction in β cell function. High levels of ICA were associated with a faster decrease in the secretion of CP (81). Aimed to modulate the immune system and prevent the destruction of β cells, studies using vaccination against GADA showed some preservation of the CP, although it did not change the needs of insulin (95,96).
There is evidence that intensive glucose control can reduce, at least temporarily, the failure of insulin secretion in T1DM. However, it is possible that the maintenance of some residual secretion facilitates the achievement of adequate metabolic control (20). The mechanisms by which intensive insulin prolongs the β-cell function in T1DM can be due to reduced glucotoxicity or by direct action in the autoimmune destruction (97).

3.2.2 Impact of maintenance of residual insulin secretion and PC

The persistence of detectable CP serum levels, especially in patients with long duration of disease, may have clinical importance. Some studies have shown that this is a prognostic factor for improved glycemic control, lower frequency of hypoglycemia, retinopathy and diabetic nephropathy (8,80,98). So far, the main information about the importance of preservation of some residual secretion in the development of chronic complications, glycemic control and incidence of hypoglycemia T1DM were obtained from the DCCT (8).

In the DCCT, the intensive glucose control significantly reduced the loss of β cell function in relation to conventional treatment. Furthermore, patients with CP ≥ 0.2 nmol / L in the intensive treatment group had lower HbA1C at baseline and through the four year follow-up period (8).

The presence of a residual capacity for insulin secretion has also been associated with a reduced risk of hypoglycemia. Data from the DCCT showed that patients with stimulated CP≥ 0.2 nmol / L for at least one year had the prevalence of hypoglycemia reduced in 30%. Among patients in intensive control group, the risk of hypoglycemia was three times lower in those who remained detectable CP than in others. In the conventional group, this difference was not observed (8).

In relation to chronic complications, the DCCT showed that patients with T1DM and undetectable levels of CP (<0.04 nmol / L) had 4.6 times greater chance of progression to diabetic retinopathy and 4.4 times greater chance of developing microalbuminuria in relation to others (8).

Some authors found no association between the frequency of chronic complications and residual β cell function. Klein and colleagues studied the relationship between serum levels of CP and severity of diabetic retinopathy in different types of diabetes in the *Wisconsin Epidemiologic Study of Diabetic Retinopathy*. Young subjects with T1DM using insulin did not present any association between CP levels and the frequency or severity of diabetic retinopathy (99). Winocour et al, on the other hand, found an association between the presence of residual secretion of CP and reduced risk of proliferative diabetic retinopathy in T1DM but no correlation to peripheral neuropathy or autonomic, hypertension, nephropathy or coronary heart disease (100).

Other studies also found no influence of CP stimulated with the development of retinopathy, neuropathy and/or microalbuminuria in T1DM. However, these studies included a small number of patients, with short time for monitoring and/or few chronic complications (100-102).

In the pathological study of Meier et al, the number of β cells found in patients with T1DM was not associated with the disease duration, but rather with glycemic control, being higher at lower blood glucose levels (90).

Today, the role of the CP only as a marker of insulin secretion is questionable. Some suggests that it may also have a direct action on target organs of chronic complications of T1DM (84). Potential actions of the CP include: improvement in nerve conduction velocity, improving the sensory and autonomic nerve function, improvement in cardiac function, decreased microalbuminuria; stimulation of the activity of nitric oxide synthase *(eNOS)* inhibition of smooth muscle cells proliferation; and decreased signal transduction of NF-kβ *(nuclear factor kappa-light-chain-enhancer of activated B cells)* with reduced inflammation. These findings have been observed *in vitro* and in clinical studies in animals and humans with T1DM (84).

3.2.3 Methods of preserving pancreatic function

The need for insulin and/or progression of pancreatic β cell damage can be avoided by preserving the ability of insulin secretion. The maintenance of some residual function, even if insufficient to avoid insulin therapy may have important advantages in better metabolic control and lower risk of chronic complications as previously described (8,84).

The DCCT showed that intensive insulin therapy can reduce the progression of β cell damage, with positive effects for up to 6 years after diagnosis (8). Brown et al in their study also confirmed this benefit (103). Intensive insulin therapy may promote survival of β cells, reducing the metabolic demand and glucotoxicity.

Immunosuppressive therapy, such as cyclosporine, azathioprine, prednisone, and anti-thymocyte globulin, aiming for depletion and inactivation of β cells, were used in newly diagnosed patients, but with limited efficacy and temporary effects due to its toxicity (104).

Immunomodulators, such as anti-CD3 monoclonal antibodies, used in newly diagnosed T1DM also allowed the maintenance of the secretion of CP for one to two years with low toxicity, and benefits in glycemic control (105-107). After 48 months of follow up, patients who received the monoclonal antibodies anti-CD3 had lower daily insulin requirements, with improved A1C and glycemic variations smaller than the control. The best results have been found in individuals under the age of 27 years and with higher CP at baseline (108).

The induction of immunological tolerance to self antigens such as GAD, insulin and oral *heat shock protein* 60 (HSP60) has been tried with controversial results in preservation of islet function (104,109,110).

Another measure that has been tested for secondary prevention of T1DM is the autologous non-myeloablative hematopoietic stem cell transplant. Voltarelli and colleagues conducted this transplant in 15 newly diagnosed patients with T1DM. Five patients have remained insulin free for up to 21 months and seven remained for more than six months without use of exogenous insulin (111). Despite the low rate of complications reported, there is a potential risk for more serious effects related to immunosuppression. (107,110). Mesenchymal stem cell therapies and combination of multiple immunomodulatory drugs are currently under study.

Several studies aiming at the preservation of β cell mass are being conducted, with a main goal: search for the cure of T1DM. The attempt to preserve β cell function even if insufficient to cure the disease, can be useful in the prevention of microvascular complications, improves glycemic control and reduced the frequency of hypoglycemic events (7,8,12,98).

In summary, we tried to emphasize some aspects of the natural history of T1DM. At first, a large mass of beta cell function is lost between the period from the surveillance diagnosis to the period of overt T1DM. Then, residual beta cell function results in better glycemic control and less microvascular complications. The rate of progression of beta cell failure may be due to several factors such as underlying genetic predisposition, age of the patient and metabolic control. Finally, CP has its role on diagnosis reserve of beta cell mass and higher levels are associated to a better glycemic control and preservation of pancreatic function. Moreover, interventions that are being held nowadays are clinically important to quality of life, mortality and morbidity of patients with T1DM.

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Part 3

Retinopathy

13

Review of the Relationship Between Renal and Retinal Microangiopathy in Type 1 Diabetes Mellitus Patients

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1. Introduction

Diabetes mellitus is a group of metabolic disorders of carbohydrate metabolism in which glucose is underutilized, producing hyperglycemia. The disease is classified into several categories. The revised classification, published in 1997 (ADA, 1997; The Expert Comitee on the Diagnosis and Classification of Diabetes mellitus, 2000), defines Type 1 diabetes mellitus (formerly known as the insulin-dependent diabetes mellitus or juvenile-onset diabetes mellitus) as a disorder caused by autoimmune destruction of pancreatic h-cells, rendering the pancreas unable to synthesize and secrete insulin. In 85–90% of cases, antibodies appear against pancreatic h-cells (ICA), acting as anti-insulin (IAA), or others such as GAD, IA-2 and IA-2h (Geiss et al 1997).

The latter complications of diabetes mellitus include both microvascular complications (predominantly retinopathy, nephropathy and neuropathy) and macrovascular complications, particularly stroke and coronary artery disease. Together, these make diabetes the seventh most common cause of death in the developed world (Geiss et al 1997).

The major microvascular complications, retinopathy and nephropathy, are the more important causes of blindness and end-stage renal disease in Europe. There are few similarities in the coexistence of DR and DN being both as microvascular disease and microscopically both have capillary basement membrane thickening. However, capillary closure is apparent in the retina and kidney after sufficient exposure to disease with duration. The pathophysiology of DN and DR are more or less similar, which commence with increase in vascular permeability. The selective increase in permeability to albumin in early DN is caused by loss of polarity across the glomerular basement membrane (Myers et al, 1982) and the disease mechanism in the eye is probably a breakdown of tight junctions between cells. The onset of proteinuria and proliferative retinopathy are both related to previous poor glycemic control, duration of diabetes and hypertension.

The detection of retinopathy is easy (by the use of fundus periodical retinographies), but the diagnosis of the early stages of nephropathy needs microalbuminuria to be determined in

urinalysis. Microalbuminuria has prognostic significance; thus, in 80% of people with Type 1 diabetes mellitus and microalbuminuria, urinary albumin excretion increases at a rate of 10–20% per year, with the development of clinical proteinuria within 10– 15 years. After the development of clinical grade proteinuria (>80%), patients go on to develop decreased glomerular filtration rate and, given enough time, end-stage renal disease (Geiss et al 1997). Several factors appear to influence susceptibility to the microvascular complications of diabetes mellitus, but our knowledge of the role and the importance of these genetic and environmental factors are still incomplete. The most powerful risk factor for microvascular complications was the duration of diabetes, but frequency of both retinopathy and nephropathy was impressively related to the level of plasma glucose at the time of examination.

From the recent studies, it is evident that the presence of retinopathy itself may reveal patients at risk for nephropathy (Estacio et al, 1998; El Asrar et al, 2002; Rossing et al, 2002; Villar et al, 1999). In a cross sectional study, patients with DR were 5.68, 13.39 and 3.51 times as likely to have DN among type1 and type2 diabetic patients (El-Asrar et al, 2002).

However, there is lack of evidence that determine the association of retinal-renal complications using the gold standard methods. The DR is characterized by microvascular abnormalities, proliferation of retinal vessels and increased retinal vascular permeability leading to the development of non-proliferative and proliferative DR, and macular edema (Williams et al 2004). The DN is a life threatening complication which predisposes to excess morbidity and mortality resulting from renal failure and cardiovascular disease (Ritz et al, 1999; Adler at al, 2003).

Our hypothesis was that the severity of DR correlates with the presence and severity of DN in people with type 1 diabetes. Studies in other populations documented a well-known association between advanced DR stages and overt nephropathy in type 1 diabetic patient (Looker et al, 2003; Gall et al, 1997). Similarly, our results provide further support to the close relationship between presence of DR and severity of DN in type1 diabetic patients.

It was reported that at least one fifth of the diabetic individuals are affected by multiple complications and the frequency increases with increasing age and duration of diabetes.

In the study of WESDR, there was a strong correlation between DN and severity of DR in all age groups (Klein et al, 1984; Klein et al 1984).

In the present study we determine the epidemiological risk factors that influence the appearance of diabetic retinopathy, and overt nephropathy, in a seventeen -year follow-up of a population sample of 112 patients who did not have diabetic retinopathy or microalbuminuria at the beginning of the study.

2. Methods

2.1 Sample size and study population

Since 1987 a register has been kept of any new cases of type I diabetes mellitus in Catalonia (Spain). The incidence of new cases over that period has been 11.4 cases per 100000 inhabitants (13.2 cases in men and 9.6 cases in women) (Castell et al, 1999).

Since 1990, there has been an ongoing registration of all diabetic patients (type I and 2) at St Joan Hospital, which is the only surgical ophthalmology centre in Reus (Spain), and having a dependent population of around 207,500 inhabitants. In 1999, there were 1495 patients with diabetes mellitus type I (Castell et al, 1999).

2.2 Design

The present study is prospective and was initiated in 1990 with 126 patients recruited with type I diabetes mellitus. The initial conditions included the absence of retinopathy and nephropathy (determined by the absence of microalbuminuria in three consecutive measures taken at one month intervals).

Two previous results were obtained at 5 and 10 years of the study (Romero-Aroca et al, 2000; Romero-Aroca et al, 2003). At the end of the study in 2007 only 112 patients were still being controlled (14 patients had dropped out during the follow up). At the end of the study (seventeen years of follow-up) the authors have determined the incidence of diabetic macular oedema and their risk factors, related to the appearance of renal overt nephropathy.

2.3 Diagnostic methods

Diabetic retinopathy was evaluated by retinal photographs through dilated pupils, of two 50^{o} fields of each eye centred firstly at the temporal to the macula and secondly at the nasal to the papilla (Aldington et al, 1995). The results were then classified into four groups (Wilkinson et al 2003):

- Mild non proliferative
- Moderate non proliferative
- Severe non proliferative
- Proliferative

Macular edema was diagnosed under stereoscopic viewing of the macula with a slit lamp and Goldmann fundus contact lens, and was considered present if we found:

- retinal thickening involving or within 500 μ m of the centre of the macula
- hard exudates at or within 500 μ m of the centre of the macula, if associated with thickening of adjacent retina (but no hard exudates remaining after retinal thickening disappeared)
- a zone or zones of retinal thickening, one disc area or larger in size, any part of which is within 1 disc diameter of the centre of the macula.

The clinical classification used was the international clinical diabetic retinopathy disease severity scale, proposed by the American Academy of Ophthalmology in 2002 (Wilkinson et al 2003). In all patients with diabetic macular oedema, a fluorescein angiography was obtained, centred on macular region to determine the leakage in that area. The fluorescein angiographic findings were categorized into three types:

- focal leakage type, which was predominantly well-defined focal areas of leakage from microaneurysm or localized dilated capillaries;
- diffuse leakage type, predominantly widespread and ill-defined leakage involving the whole circumference of the fovea;
- cystoid leakage type, predominantly diffuse leakage but with pooling of dye in the cystic spaces of the macula in the late phase.

Since 2000, all patients with diabetic macular oedema have been given an optical coherence tomography OCT), repeated every 4 months as a control test. Optical coherence tomography was performed with a OCT model TOPCON TRC NW 7SF. The retinal map algorithm uses measurements along 6 radial lines, 6 mm in length, to produce a circular plot in which the foveal zone is the central circular zone of 1.00 mm in diameter. Macular edema measured by OCT was defined as a retinal thickening of more than 216 microns, and was classified as follows, using the Otani et al patterns amplified by the two tractional forms

described later (Otani et al, 1999): Sponge-like retinal thickness, defined as increased retinal thickness with reduced intra retinal reflectivity and expanded areas of lower reflectivity; cystoid macular oedema, characterized by the intra retinal cystoid spaces at the macular area; serous retinal detachment was thought to be present if the posterior surface of the retina was elevated above the outer border of the highly reflective band, regarded as the signal generated mainly by the retinal pigment epithelium. Only patients with a visible separation between the layer of photoreceptors and the pigment epithelium, was classified as serous detachment; if we observed the photoreceptor layer adjacent to pigment epithelium, we classified the case as cystoid macular oedema.

2.4 Inclusion criteria

Patients with type I diabetes mellitus (insulin dependent or young-onset diabetes mellitus)

2.5 Exclusion criteria

Presence of diabetic retinopathy at the beginning of study, presence of diabetic nephropathy at the beginning of study, presence of microalbuminuria at the beginning of study, patients with LADA diabetes (latent autoimmune adult diabetes), patients with type 2 diabetes mellitus (not insulin-dependent or older-onset diabetes mellitus), patients with type 2 diabetes mellitus appeared before 30 years of age (MODY diabetes mellitus)

2.6 Definition of variables

Visual acuity in each eye was measured on the Snellen chart and recorded as a decimal value, with best refraction for distance. All data manipulations were performed on visual acuities expressed in log MAR form. The legal blind subject was defined as corrected visual acuity less than or equal to 0.1 in the better eye; reduced visual acuity as less than or equal to 0.4 and greater than 0.1 in the better eye.

The epidemiological risk factors included in the study were:

- Gender and current age.
- Duration of diabetes mellitus, classified in the statistical study in two groups: below 20 years of duration and equal to or more than 20 years duration.
- Type of diabetic retinopathy classified into two groups: first with a diabetic retinopathy lower than severe pattern, and the second with patients with severe or proliferative pattern, patients who need scattered photocoagulation were classified in tis second group.
- Arterial hypertension, which indicates a systolic measurement above or equal to 140 mm Hg and the diastolic measurement above or equal to 90 mm Hg, or when the patient is taking anti-hypertensive medications.
- Levels of glycated haemoglobin (HbA1c) as recommended i by the American Diabetes Association (ADA, 1997) as the major component of HbA1c (accounting for 80% of HbA1c), was measured every 3 months. The control of glycaemia was considered in concordance with the European Diabetes Policy Group, into two groups of patients i.e. over or under 7.0% (European Diabetes Policy Group, 1999). The value included in the statistical analysis was the mean of all values obtained over the 15 years.
- Presence of microalbuminuria, defined as increased albumin excretion (30-300 mg of albumin/24 h or 20-200 μg/min of creatinine) on two of three tests repeated at intervals of 3-6 months as well as exclusion of conditions that invalidate the test (Geiss et al,

1997). The test was performed annually. After microalbuminuria was diagnosed, repeated testing was made within a period of 3-4 months.

- Presence of diabetic nephropathy, defined as clinical albuminuria or overt nephropathy by the American Diabetes Association, corresponding to protein excretion >300 mg/24h (>200 μg/min or >300 μg/mg of albumin: creatinine ratio). Measurement of creatinine clearance as an index of glomerular filtration rate was performed on the same urine collection (Geiss et al, 1997).
- Patients were classified as having macro vascular disease if one or more of the following were present: symptoms of angina pectoris, history of myocardial infarction, coronary artery by pass grafting, percutaneous tranluminal coronary angioplasty, symptoms of or operation for intermittent claudication, history of amputation, transient ischemic attack, stroke.
- Levels of triglycerides and fractions of cholesterol (HDL-cholesterol and LDL-chorlesterol). In the statistical analysis, we classified the patient into normal or higher values, according to the ADA categories as patients with high risk if LDL-cholesterol 3.35 mmol/L (130 mg/dl), HDL-cholesterol 0.90 mmol/L for men and >1.15 mmol/L for women (35 mg/dL for men and 45 mg/dL for women), and triglycerides 1.5 mmol/dL (400 mg/dL), (Expert Panel on Detection, Evaluation, And Treatment of high Blood Cholesterol in Adults, 2001).

2.7 Statistical methods

All statistical analyses were carried out using the SPSS software package (version 18.0), results are expressed as mean±standard error, a P-value of less than 0.05 was considered to indicate statistical significance.

Differences between those included in analyses were examined using the two sample Student T-tests or one-way ANOVA, for continuous or quantitative data, as visual acuity or current age. For the qualitative or categorical data we used the Chi-square test in the univariate phase of study, with determination of Odds ratio for each variable.

The Kruskal-Wallis test and the least significant difference test using ranks for multiple comparisons were carried out to evaluate the correlation between best-corrected visual acuity an OCT findings.

In the multivariate phase of analysis the relationship of diabetic retinopathy, microalbuminuria and overt nephropathy, to various demographic and other risk factors were examined using logistic regression analysis; the full model was built including gender and age a priori.

3. Results

Demographic variables of the patients

Gender: 54 patients were men (48.2%) and 58 were women (51.8%)

The mean of age was 39.94 ± 10.53 years old (24 – 61 years), the mean of diabetes mellitus type I duration was 23.42 ± 7.57 years (12 – 45 years). The arterial hypertension was present in 44 patients (39.3%).

The means of the different quantitative data were:

- Glycosylated haemoglobin A_{1c}: 7.69% ± 1.24 (4.50% – 11.40%)
- LDL-Cholesterol: 3.50 ± 0,57 mmol/l (3.00 – 4.00)

- HDL-Cholesterol: 1.12 ± 0,42 mmol/l (0.70 – 2.02)
- Triglycerides: 1.47 ± 0.76 mmol/l (0.90 – 3.00)

Visual acuity study

Mean visual acuity after 17 years was 0.77 ± 0.34 (0,02 – 1) in the Snellen chart test; and +0.37 ± +0.72 (+ 1.7 - + 0) in the Log MAR test.
Low vision (defined as vision in the best eye >0,1 and < 0.4 in the Snellen chart) was detected in 13 patients (11.6%) and blindness (AV < 0.1 in Snellen chart) in 14 patients (12.5%).

Incidence of diabetic retinopathy (Table 1)

After 17 years there were 62 patients (55.4%) with different types of diabetic retinopathy. The Rate of progression was 8.30 person-year.

- Mild diabetic retinopathy in31 patients (27.7%)
- Moderate diabetic retinopathy in 7 patients (6.3%)
- Severe diabetic retinopathy in 5 patients (4.5%)
- Proliferative diabetic retinopathy in 18 patients (16.1%)

There were 23 patients (20.5%) with diabetic macular edema after 17 years, the Rate of progression was 3.08 person-year. The mild or moderate form of diabetic macular edema was present in 13 patients (11.6%) and the severe form of macular edema in 10 patients (8.9%).
In the 62 patients with diabetic retinopathy 17 (27.42%) developed overt nephropathy, with a rate of progression 4.11 person-year. In patients with proliferative form of diabetic retinopathy (23 patients) 11 developed microalbuminuria (47.82%), the rate of progression was 7.17 person-year.

Statistical study of diabetic retinopathy

Univariate study with the application of chi squared test (Table 1).
The factors significant in the appearance of diabetic retinopathy were as follows: duration of diabetic retinopathy p<0.001, presence of arterial hypertension p<0.001, levels of glycated haemoglobin (HbA_{1c}) > 7.5% p<0.001, high levels of LDL-cholesterol p<0.001, high levels of tryglicerides p=0.003 and presence of overt nephropathy p= 0.001.

Logistic regression of diabetic retinopathy (Table 2).

The followings factors studied were significant in the appearance of diabetic retinopathy: Duration of diabetes mellitus more than 20 years p< 0.001, presence of arterial hypertension p<0.001, high levels of HbA_{1c} p< 0.001, high levels of tryglicerides p=0.004, high levels of LDL-Cholesterol p= 0.002, and overt nephropathy p=0.021.

Statistical study of diabetic nephropathy

Univariate analysis with the application of chi squared test(Table 1).
The factors significant in the apparition of overt nephropathy were: presence of arterial hypertension p<0.001, high levels of HbA_{1c} p<0.001, high levels of LDL-Cholesterol p=0.010, high levels of triglycerides p=0.003, and presence of diabetic retinopathy p=0.021. When we introduced the presence of proliferative diabetic retinopathy against the presence of any retinopathy, the chi squared test had a result of p<0.001, and for proliferative diabetic retinopathy p< 0.001.

Logistic regression of diabetic nephropathy (Table 2).

The significant factors were: the presence of arterial hypertension p<0.001, and high levels of HbA_{1c} p<0.001, high levels of LDL-Cholesterol p=0.002, high levels of triglycerides p=0.009,. Also the presence of diabetic retinopathy was significant p=0,021. When we introduced the presence of proliferative diabetic retinopathy against the presence of any retinopathy, the chi squared test had a result of p<0.001

	Diabetic retinopathy				Overt Nephropathy			
Risk factor	Chi square		Logistic regression		Chi square		Logistic regression	
	Signifi-cance (p)	Odds ratio	Signifi cance (p)	Odds ratio	Signifi-cance (p)	Odds ratio	Signifi-cance (p)	Odds ratio
Gender	0.237	1.397	0.829	0.881	0.743	0.107	0.870	0.910
Glycated haemoglobin (HbA_{1c}) >8%)	<0.001	11.011	<0.001	5.575	<0.001	55.687	<0.001	38.360
Arterial hypertension	<0.001	28.193	0.007	6.579	<0.001	12.777	0.023	10.271
Duration of diabetes mellitus (20 years)	<0.001	33.623	<0001	12.096	0.913	0.012	0.170	0.394
HDL-Cholesterol	0.828	0.047	0.813	0.837	0.019	5.201	0.061	0.195
LDL-Cholesterol	<0001	18984	0.002	10.304	0.010	2.715	0.002	3.555
Triglycerides	0.003	8.442	0.004	1.528	0.003	3.513	0.009	2.912
Overt Nephropathy	0.001	6.097	0.021	3.498				
Retinopathy					0.021	6.097	0.021	2.153
Proliferative diabetic retinopathy					<0.001	14.814	<0.001	4.306

Table 1. Chi squared and logistic regression analysis for diabetic retinopathy and microalbuminuria.

Patients only with retinopathy	Patients only with overt nephropathy	Patients with retinopathy and overt nephropathy
Duration of diabetes mellitus (4.679)*	High levels of HbA1c (7.250)*	High levels of HbA1c (6.471)*
High levels of HbA1c (2.250)*	High levels of triglycerides (1.713)*	High levels of triglycerides (2.810)*
Arterial hypertension (2.668)*	Duration of diabetes mellitus (1.029)*	Arterial hypertension (2.657)*
High levels of LDL-cholesterol (1.277)*	Arterial hypertension (0.742)*	Duration of diabetes mellitus (2.269)*
High levels of triglycerides (1.254)*	High levels of LDL-cholesterol (-1.360)*	High levels of LDL-cholesterol (1.232)*

* = Function of classification coefficients.

Table 2. Fisher's classification coefficient.

Statistical application of discriminate analysis

At the end of the study we may observe that four groups of patients had been formed:

- those without any form of microangiopathy (overt nephropathy or retinopathy) 45 patients (group A).
- those with only retinopathy (45 patients) (group B).
- those with only overt nephropathy (5 patients) (group C).
- those with both overt nephropathy and retinopathy (17 patients) (group D).

In this case we needed to apply a discriminate analysis to evaluate the risk factors for the different groups.

Applying Fisher's coefficient indicated that (Table 2):

- for group B the risk factors were: duration of diabetes mellitus (4.679), high levels of HbA_{1c} (2.250) and high levels of LDL-Cholesterol (2.268)
- for the group C only the high levels of HbA_{1c} (7,250) were highly correlated
- for the group D the significant factors were: high levels of HbA_{1c} (6,471), presence of arterial hypertension (2.657), high levels of triglycerides (2.810) and duration of diabetes mellitus (2.269)

4. Discussion

The diabetic retinopathy (DR) and diabetic nephropathy (DN) are the two major complications of diabetes mellitus. The proliferative diabetic retinopathy and proteinuria secondary to DN are both late complications of diabetic overt nephropathy, these usually occur 10 to 15 years after the onset of type 1 DM, and are strongly associated with each other. The epidemiology of diabetic overt nephropathy and retinopathy in type 1 DM, are different, thus for diabetic overt nephropathy increases its prevalence since 10% at ten year duration of diabetes mellitus, achieving the highest value after 40 years with a 40% of diabetic patients with nephropathy, since this point the curve levels off, and only a minority of patients develop clinically significant renal abnormalities, and patients who survive 35 years of type 1 DM without developing DN are at extremely low risk of doing so in the future. Against this curve the diabetic retinopathy succeed in a different form in type 1 diabetic patients, thus the diabetic retinopathy is rare before 10 years DM duration, and

increases the prevalence, since this point to a levels upper 80% after 20 years diabetes duration, without a decreases after these 20 years of duration, as we observed in our study.
The incidence of DR was 55.4%, and was lower than in other studies such as Klein et al 1998 (Klein et al, 1998), but this may be because the sample studied did not present diabetic retinopathy at the beginning and patient controls were stricter than in the rest of the patients with diabetes mellitus type I (controls every 3 months), we could concluded that the mean level of HbA_{1c} was 7.69% ± 1.24 (4.50% – 11.40%) is better than the achieved by WESDR (Klein et al, 1998). The diabetic macular edema appeared in 20.5% of patients, which was more similar to the findings in the Klein study at 14 years (26%). In the present study the incidence of diabetic macular edema is higher than the proliferative form of diabetic retinopathy in type I diabetes mellitus patients, as was observed in other studies.
With regard to nephropathy diabetes accountings for more than 19.6% of all cases of DN. The DN presents initially as intermittent microalbuminuria that progresses to persistent microalbuminuria, and is accompanied by a decline in the glomerular filtration rate. These dates were in agreement with other studies published in our country on renal failure in diabetes mellitus type I patients Smatjes et al (Esmatjes et al, 1998) found an incidence of 44.5% with some form of renal failure at 20 years in type I diabetes mellitus.
The relationship between DN and DR was well described, thus the Wisconsin Epidemiologic Study of Diabetic Retinopathy (Klein et al, 1993) associated the presence of gross-proteinuria at baseline examination with a 96% increase in the risk of progression to proliferative retinopathy. Also in the Steno study (Kofoed-Enevoldsen et al, 1987), people with type I diabetes mellitus and gross-proteinuria at baseline had an increase risk of progression to proliferative retinopathy (12% annually) compared to those without proteinuria (1%-2% annually).
At the end of our study we can see that four groups of patients had formed: those without overt nephropathy or diabetic retinopathy (45 patients), patients with only overt nephropathy (5 patients), those with only diabetic retinopathy (45 patients), and those with overt nephropathy and diabetic retinopathy (17 patients). The statistical test used for examining these data was a discriminate test, which allowed us to identify the risk factors that influence any of these groups.
In the group of patients with only DR the duration of diabetes mellitus was the more important risk factor and for the group with Dr and DN the most important risk factor is the high levels of HbA_{1c}.
We may assume then, that for the development of only retinal lesions in diabetes mellitus, the duration of the disease is the most important followed by and in a second level of importance the levels of HbA1c and arterial hypertension; and for the development of renal and retinal lesion simultaneously poor control of glycaemia measured by levels of HbA1c were more important than the duration of diabetes mellitus.
Two broader groups of patients can be assumed to have been formed in this study, the first being those patients who developed only diabetic retinopathy, and the second those with both diabetic retinopathy and renal lesion (overt nephropathy). This conclusion is consistent with previous studies as that of Lövestam-Adrian in 1998 (Lövestam-Adrian, et al, 1998), in which after a 10-year follow-up of a population of 24 patients, with proliferative diabetic retinopathy at the beginning of the study, only two developed microalbuminuria, That study concluded that there are, at least partly, different pathogenic mechanisms behind diabetic retinopathy and overt nephropathy.

5. Conclusion

Despite there being a poor relationship between overt nephropathy and diabetic retinopathy ($p=0.021$ in the present study), the presence of overt nephropathy correlated well with severe forms of diabetic retinopathy (as proliferative Dr $p<0.001$ in the present study); and in addition, at the end of study two broad group of patients had been configured, the first those who developed only diabetic retinopathy, and the second with diabetic retinopathy and renal lesion (overt nephropathy). For the first group with only DR , duration of diabetes mellitus is the most important risk factor, and for the second group (patients with DR and DN) the levels of HbA_{1c} and blood pressure are the most important.

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14

Ocular Complications of Type 1 Diabetes

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1. Introduction

Type 1 diabetes is a complex metabolic disease involving multiple organ systems which may cause severe visual impairment. Almost all parts of the eye may be affected including: the extra-ocular muscles, intra-ocular lens, the optic nerve, and retina.
Diabetes is the leading cause of blindness between the ages of 20 and 74 in many developed countries (Cheung et al., 2010; Powers, 2008). Individuals with diabetes are 25 times more likely to become legally blind than individuals without diabetes. Blindness is primarily the result of diabetic retinopathy that accounts for ¼ of blind registrations in the western world (Cheung et al., 2010; Powers, 2008).
Prevention of severe visual impairment in type 1 diabetes includes: optimal glycemic control, the treatment of ancillary risk factors such as hypertension, regular ophthalmic screening, and early diagnosis and treatment of ocular complications.
In the following chapter we will describe the ocular complications of diabetes and the treatments for these conditions.

2. Extra-ocular muscles

Diabetics may present with a sudden onset of diplopia (double vision). This is usually caused by the partial or complete paralysis of one of the extra-ocular muscles due to microvascular damage to the third, fourth or sixth cranial nerve (Thomas & Graham, 2008; Kline et al. 2010).
When a third cranial nerve palsy occurs, it is important to differentiate between a diabetic nerve palsy and paresis due to compression of the nerve from an aneurysm at the junction of the posterior communicating and internal carotid arteries. In diabetic nerve palsy the pupil is often spared meaning that it continues to react to light appropriately despite damage to the motor capabilities of the third cranial nerve. Typically in cranial nerve palsy due to a space occupying lesion such as an aneurysm, the pupil in the affected eye is dilated. In 20% of patients with diabetic nerve palsy there may be pupil involvement, however this is usually a mild efferent defect (Kline et al. 2010). Aneurysms are rare in children but may be present in adolescents, so ruling it out, by neuroimaging, is crucial. Pain may also be present in diabetic third nerve palsy. When there is paresis of the fourth or sixth cranial nerve, referral to a neuro-ophthalmolgist is also recommended for follow-up and to exclude other causes, such as myasthenia gravis or brain lesions.

When the oculomotor defect is due to microvascular complications of diabetes the prognosis is good.
Recovery of ocular motor function generally begins within three months of onset and recovery is usually complete. Although the diplopia can be debilitating, due to the generally limited course of these complaints, patients can usually be effectively managed conservatively with eye patching. Surgery is rarely indicated.

3. Lens: Cataract and refractive changes

Hyperglycemia can reduce lens clarity. Hyperglycemia also induces changes in the refractive index and accommodative amplitude of the lens, both of which also act to reduce visual function (Flynn & Smiddy, 2000).

3.1 Refractive and accommodative changes

One of the most frequently encountered ocular manifestations of diabetes is abrupt changes in the refractive power of the lens. When the blood glucose level is high, the glucose concentration in the aqueous humor, the fluid surrounding the lens, increases as well. This causes the glucose concentration in the lens to increase by diffusion (Flynn & Smiddy, 2000). Under normal conditions glucose is metabolized inside the lens by glycolysis. However, when the glucose level in the lens is very high, glycolysis enzymes are overridden and some glucose is reduced by the enzyme aldose reductase and converted to sorbitol (Stirban et al., 2008). Sorbitol is metabolized slowly by the lens cells and accumulates, increasing the osmotic pressure inside the lens. This increased osmotic pressure leads to an influx of water from the aqueous humor and the lens swells. This larger lens is more convex and therefore more powerful at bending incoming light which alters the focal point of the eye, causing acute nearsightedness (myopia) and blurred distance vision.
These refractive changes may be up to three to four diopters and may last for several weeks. Thus, patients with poorly controlled blood glucose levels experience transient refractive changes due to fluctuating levels of glucose in the blood. Acute blurring of vision may be the first symptom of undiagnosed or poorly controlled diabetes.
Accommodation, the ability to adjust focus for near tasks such as reading, is also affected in patients with diabetes. Studies have shown that diabetics have decreased amplitude of accommodation compared to age matched controls, and require spectacle correction for near work at a younger age than non-diabetics (Flynn & Smiddy, 2000).

3.2 Cataract

Cataract is a common cause of visual impairment in patients with diabetes. Epidemiological studies have revealed an up to five-fold increased prevalence of cataracts in diabetic patients. Individuals with type 1 diabetes manifested a greater prevalence of cataracts between the ages of 18 to 44 than age-matched controls (Obrosova et al., 2010). Duration of diabetes and quality of glycemic control are the major risk factors for early cataract development.
Potential mechanisms of diabetic cataract formation include accumulation of lenticular sorbitol, as described in the previous section (3.1). This reduces lens clarity leading to early cataract formation. It has also been postulated that recurrent high levels of glucose in the lens lead to the glycolation of lens proteins from increased non-enzymatic glycation and

oxidative stress to the lens (Obrosova et al., 2010). This causes diabetic patients to develop age related lens changes similar to non-diabetic age related cataracts, except at a younger age than non-diabetics (Bobrow et al. 2010). Several studies have analyzed the effect of vitamin and anti-oxidant supplements, such as vitamin C, E and beta carotene and zinc on preventing or slowing progression of age related cataracts in diabetes without showing any statistically significant benefit with their use (AREDS report no. 9, 2001 as cited in Obrosova et al., 2010).

A rarer form of cataract in diabetics that is seldom encountered in clinical practice today is called the 'true diabetic cataract'. This is typically seen in young patients with uncontrolled diabetes. Any rapidly maturing (i.e whitening) cataract in a child or a young adult should raise awareness to the possibility of diabetes.

Cataract surgery is indicated when visual function is significantly impaired by the cataract. Surgery is also indicated if the cataract obscures the view of the retina and makes the diagnosis and treatment of diabetic retinopathy difficult. Cataract surgery is safe in diabetic patients and there is a 95% success rate in terms of improved visual acuity (Obrosova et al., 2010). Good glycemic control, fluid and electrolyte balance should be maintained perioperatively and the patient's primary care physician and anesthesiologist should be involved. It is recommended that the surgery be scheduled in the morning to minimize changes in the patient's usual schedule (Purdy et al., 2010). Some controversy exists regarding a potential association between cataract surgery and a subsequent worsening of diabetic retinopathy. Patients should be made aware of this risk pre-operatively. Cataract surgery and its effect on diabetic retinopathy will be discussed in more detail the diabetic retinopathy section (7.6.2).

4. Cornea

Structural changes to the corneal basement membrane in diabetes decrease the adhesion of corneal epithelial cells to the deeper stromal tissue. This increases the risk of recurrent corneal erosions (Reidy et al., 2010). In addition, accumulation of sorbitol in the cornea during periods of hyperglycemia leads to hypoesthesia (a loss of corneal sensation). Both hypoesthesia and epithelial adhesion dysfunction occur more frequently with increased severity and duration of diabetes. In these patients, any epithelial injury, either from trauma, during ocular surgery or from routine contact lens use, may result in prolonged healing times. This increases the risk of severe complications such as bacterial infiltration and ulceration.

5. Iris and pupil

Bilateral tonic pupils may be seen in diabetic patients (Kline et al., 2010). This manifests with sluggish, segmented pupillary reactions to light and better response to near effort, followed by slow redilation of the pupil. Tonic pupils are caused by microvascular damage to postganglionic parasympathetic pupillomotor nerve fibers. Diminished pupillary response is also seen due to glycogen infiltration of the pigment epithelium and sphincter and dilator muscles (Thomas & Graham, 2008).

Rubeosis iridis, neovascularization in the iris, is a serious complication of diabetes which occurs in patients with severe diabetic retinopathy (Thomas & Graham, 2008). Growth factors released from the ischemic retina induce the development of intertwining blood

vessels on the anterior surface of the iris (figure 1). These vessels can block the normal drainage of fluid from the anterior chamber, leading to a sharp and persistent rise in intraocular pressure. This complication is known as neovascular glaucoma. This type of glaucoma is often refractory to treatment and can be associated with pain from very high ocular pressure. Topical medical therapy used commonly in other forms of glaucoma is often less effective. Treatment should include aggressive control of the underlying retinal disease with peripheral laser ablation to reduce ischemia. The treatment of proliferative diabetic retinopathy will be discussed in more detail in section 7.6.2.3.

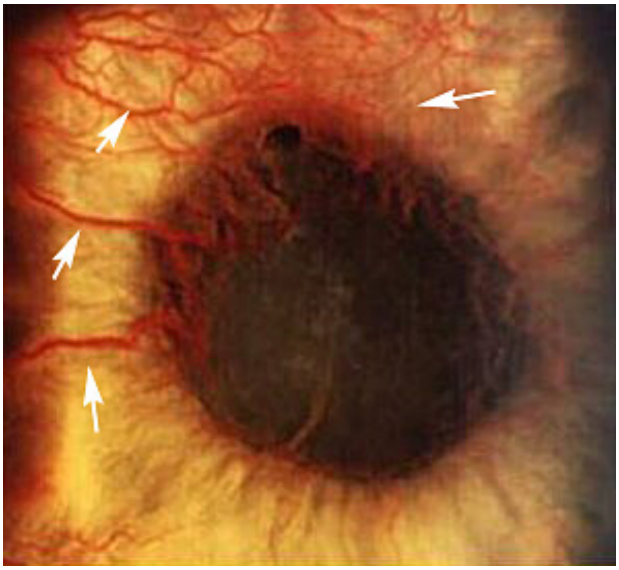

Fig. 1. Neovascularization of the Iris: These pathologic blood vessels on the iris (white arrows) are associated with retinal ischemia in proliferative diabetic retinopathy. The normal iris has no visible surface blood vessels.

6. Optic nerve

6.1 Diabetic papillopathy

In diabetic papillopathy, chronic swelling of the optic disc often associated with mild visual impairment. The suspected cause is mild reversible ischemia of the optic nerve head (Ostri et al., 2010; Kline et al., 2010). Risk factors include pronounced recent decrease in hemoglobin A_{1C} and a small cup to disc ratio of the optic nerve head. Patients often present with no visual complaints or with a mild nonspecific visual disturbance such as mild distortion or blurring. There is no pain and visual acuity is usually normal but may be slightly diminished. There is no afferent pupillary defect. An enlarged blind spot is seen on visual fields. Clinical examination reveals unilateral or bilateral hyperemic edema of the optic disc, accompanied by dilation of inner disc surface vessels, vascular leakage and axonal swelling (cotton wool spots). These enlarged vessels may be confused with neovascularization of the disc but these radially dilated vessels do not extend into the vitreous (Figure 2).

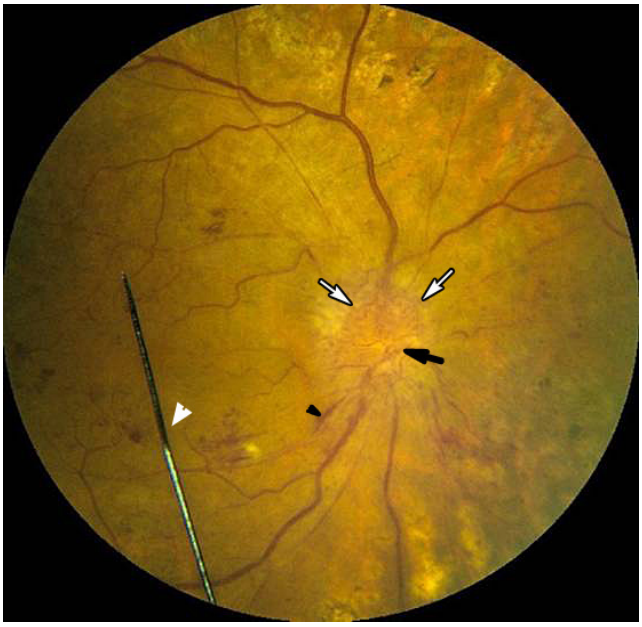

Fig. 2. Diabetic Papillopathy. A chronic swelling of the optic disc often associated with mild visual impairment. This color fundus photograph shows blurred disc margins (white arrows), flame shaped hemorrhages (black arrowhead). The dilated blood vessels on the optic disc (black arrow) may be confused with neovscularization but are radially dilated, do not enter the vitreous cavity and do not leak on fluorescein angigraphy.[marked by the white arrowhead the pointer aiding the patient's fixation during photography].

When diabetic papillopathy is suspected, it is important to perform fluorescein angiography. In diabetic papillopathy, dye leakage is limited to the disc and peripapillary retina as opposed to the intravitreal leakage seen in the case of neovascular lesions. Diabetic retinopathy is usually present at diagnosis, but in 20% of reported cases there was no clinical evidence of any diabetic retinopathy (Kline et al., 2010). If the optic disc edema is bilateral, the initial evaluation should include brain imaging and lumbar puncture to rule out intracranial space occupying lesions and elevated intracranial pressure.

The optic disc edema resolves in many cases without treatment, usually within two to ten months. Minimal optic atrophy is seen in 20% of cases. In rare cases, especially in poorly controlled patients, diabetic papillopathy may progress to non-arteritic anterior ischemic optic neuropathy (NAION) with significant optic atrophy and arcuate visual field defects. In most cases, long term visual acuity depends on the associated diabetic retinopathy. There is no proven treatment for diabetic papillopathy.

6.2 Non-arteritic anterior ischemic optic neuropathy (NAION)

Non-arteritic anterior ischemic optic neuropathy (NAION) occurs as a consequence of the interruption of blood flow to the optic nerve at the level of the optic disc. NAION is characterized by diffuse or segmental, hyperemic or pale optic disc edema. It is usually

unilateral, and in contrast to diabetic papillopathy, damage to the optic nerve ganglions causes decreased visual acuity and/or visual field loss. Vision is usually not worse than 20/200 and the typical visual field defect is altitudinal. Diabetes is a risk factor for NAION and the condition may present at a younger age in diabetic patients (Kline et al., 2010).
After an episode of NAION visual acuity may be stable but can also decline slowly over weeks to months until eventual stabilization. The initial finding upon examination is optic disc swelling, but this resolves over time and is replaced by optic disc atrophy within 4-8 weeks. There is no proven treatment for this condition.

7. Diabetic retinopathy

Damage to the retinal capillaries and other small vessels is the hallmark of diabetic eye disease and is known as diabetic retinopathy. This condition is the major cause of blindness and visual disability in patients with type 1 diabetes.

7.1 Epidemiology

Diabetic retinopathy is one of the most frequent causes of blindness in working aged adults (20-74 years) (Regillo et al., 2010; Cheung et al., 2010). In the USA an estimated 86% of patients with type 1 diabetes have some degree of diabetic retinopathy. Data from the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) showed that within 5 years of diagnosis of type 1 diabetes, 14% of patients developed retinopathy, with the incidence rising to 74% by 10 years (Klein et al., 2008; Varma, 2008). In people with retinopathy at the WESDR baseline examination, 64% had their retinopathy worsen, 17% progressed to proliferative diabetic retinopathy (PDR) and about 20% developed diabetic macular edema during 10 years of follow-up.
The WESDR data in type 1 diabetics showed that 25 years after diagnosis, 97% of patients developed retinopathy, 43% progressed to PDR, 29% developed diabetic macular edema and 3.6% of patients younger than 30 at diagnosis were legally blind (Klein et al., 2008). Fortunately, recent advances in glycemic control, ophthalmic treatment and patient education seem to be working. The WESDR results also showed a reduction in the yearly incidence and progression of diabetic retinopathy during the past 15 years (Varma 2008).
The course of diabetic retinal disease in children is fairly benign. Severe complications such as proliferative diabetic retinopathy are uncommon in children before puberty (Raab et al., 2010).

7.2 Risk factors

Several risk factors influence the development and progression of diabetic retinopathy. The following list contains most of the important risk factors known today.

1. Diabetes duration: The longer the duration of diabetes, the higher the risk of developing diabetic retinopathy and of having a severe manifestation of the disease. (Simon et al., 2010; Cheung et al., 2010).
2. Hyperglycemia: Good glycemic control has been shown to significantly prevent the development and progression of diabetic retinopathy. Every 1% decrease in hemoglobin A_{1C} leads to a 40% reduction in the risk of developing retinopathy, a 25%

reduction in the risk of progression to vision threatening retinopathy and a 15% reduction in the risk of blindness (Cheung et al. 2010, DCCT group, 1995).

3. Hypertension: Good blood pressure control is important in reducing the risk of retinopathy. Every 10 mmHg reduction in systolic blood pressure leads to a reduction of 35% in the risk of retinopathy progression and a reduction of 50% in the risk of visual loss (Cheung et al. 2010).
4. Hyperlipidemia: High cholesterol may also be a risk factor for diabetic retinopathy progression (Cheung et al., 2010).
5. Genetic factors: The Diabetes Control and Complications Trial (DCCT group, 1997) showed a heritable tendency for developing diabetic retinopathy, regardless of other risk factors.
6. Ethnicity: Diabetic retinopathy in America is more prevalent among African Americans, Hispanic and south Asian groups than in Caucasians with otherwise similar risk profiles (Cheung et al., 2010).
7. Pregnancy: Pregnancy is associated with worsening of diabetic retinopathy (DCCT group, 2000). All pregnant women need to be closely monitored throughout pregnancy. Pregnancy in type 1 diabetes is discussed in further detail in section 7.6.1.

7.3 Pathophysiology

The normal retina has a blood–retinal barrier (BRB) which consists of cells that are tightly joined together to prevent certain substances from entering the retinal tissue. An important part of the BRB is the non-fenestrated capillaries of the retinal circulation. In diabetic retinopathy, damage to retinal blood vessels leads to a breakdown of the BRB and the leakage of fluid, blood and protein into the retinal tissue.

Diabetic retinopathy is induced when hyperglycemia and other causal risk factors trigger a cascade of biochemical changes leading to microvascular damage in the retina. Hyperglycemia leads to rise of sorbitol concentrations via the action of aldose reductase. This process increases oxidative stress by reducing intracellular levels of reduced glutathione, an important antioxidant (Stirban et al., 2008). Intracellular hyperglycemia also increases synthesis of diacylglycerol an activating cofactor for protein kinase C (PKC). Activated PKC decreases the production of anti-artherosclerotic factors and increases production of pro-artherogenic factors, pro-adhesive and pro-inflammatory factors (Stirban et al., 2008). Hyperglycemia also leads to accumulation of advanced glycated end products which are pro-inflammatory and activate PKC (Stirban et al., 2008). Intracellular hyperglycaemia increases intracellular N-acetylglucosamine levels. This byproduct reacts with serine and threonine residues in transcription factors, resulting in pathologic changes in gene expression (Stirban et al., 2008). The final consequence of these pathological processes is increased inflammation and increased oxidative stress which cause endothelial cell dysfunction in retinal blood vessels.

Endothelial cell dysfunction induces retinal arteriolar dilatation which increases capillary bed pressure. This results in microaneurysm formation, vessel leakage and rupture (Cheung et al., 2010). Vascular permeability is also increased from loss of pericytes and increased endothelial proliferation in retinal capillaries. The breakdown of the blood-retinal barrier allows fluid to accumulate in the deep retinal layers where it damages photoreceptors and other neural tissues. This is the mechanism by which macular edema reduces visual acuity.

In some capillaries there is endothelial cell apoptosis. Vessels become acellular leading to vascular occlusion and non-perfusion of local retinal tissue (Stirban et al., 2008). The resultant retinal ischemia promotes the release of inflammatory growth factors, such as vascular endothelial growth factor (VEGF), growth hormone- insulin growth factor and erythropoietin (Cheung et al., 2010). These factors influence neovascularization, the proliferation of new capillaries, which is the hallmark of proliferative diabetic retinopathy.

7.4 Classification and clinical features

Diabetic retinopathy is classified into two stages: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). In NPDR the vascular changes occur within the retina and do not cross the retinal surface. The more advanced stage of PDR is marked by neovascularization wherein new blood vessels grow out from the retinal surface towards the vitreous cavity.

A major cause of vision loss in diabetic retinopathy is diabetic macular edema (DME). DME occurs when leaky capillary beds allow fluid to accumulate in the part of the retina responsible for central vision. This edema can occur in patients with any stage of underlying retinopathy from mild NPDR to severe PDR.

Visual impairment is usually related to the state of macular disease and the consequences of neovascularisation such as vitreous hemorrhage and retinal detachment. As such, the level of retinal disease does not always correlate with visual function and severe diabetic retinopathy can be present initially without significant visual loss.

7.4.1 Non-proliferative diabetic retinopathy (NPDR)

In NPDR the retinal microvascular changes occur within the retina and do not extend beyond the surface of the retina. The patient with NPDR is usually asymptomatic and visual acuity is preserved unless the macula is affected.

Clinical findings include microaneurysms (saccular enlargements of weakened capillaries), intra-retinal hemorrhages, hard exudates (lipid filled macrophages), cotton wool spots (nerve fiber layer infarcts)(figures 3-4), venous beading (focal venous dilatations and constrictions) and intra-retinal microvascular abnormalities (IRMA's, dilated pre-existing capillaries) (Regillo et al., 2010, Cheung et al., 2008).

Fluorescein angiography (FA) is an essential tool for evaluating the retinal circulation and retinopathy stage. Sodium fluorescein is injected into the systemic circulation, and an angiogram is obtained by photographing the fluorescence emitted after illumination of the retina. In NPDR, the FA shows microaneurysms as dye filled outpouchings. Hemorrhages appear as black dots as the blood obscures the fluorescence from the retina and choroid below (figure 5).

NPDR is classified as mild, moderate or severe, reflecting the risk of progression to PDR (Table 1) as determined by the Early Treatment in Diabetic Retinopathy Study (ETDRS) (ETDRS group, 1995). The diagnosis of severe NPDR is made when one of three findings is present: diffuse intra-retinal hemorrhages and microaneurysms in all 4 retinal quadrants, venous beading in 2 quadrants or one intra-retinal microvascular abnormalities anywhere. Fifteen persent of patients with severe NPDR will progress to high-risk PDR within 1 year. When any two features of severe NPDR are present, the patient is said to suffer from very severe NPDR and the one year risk of progression to high risk PDR increases to 45%.

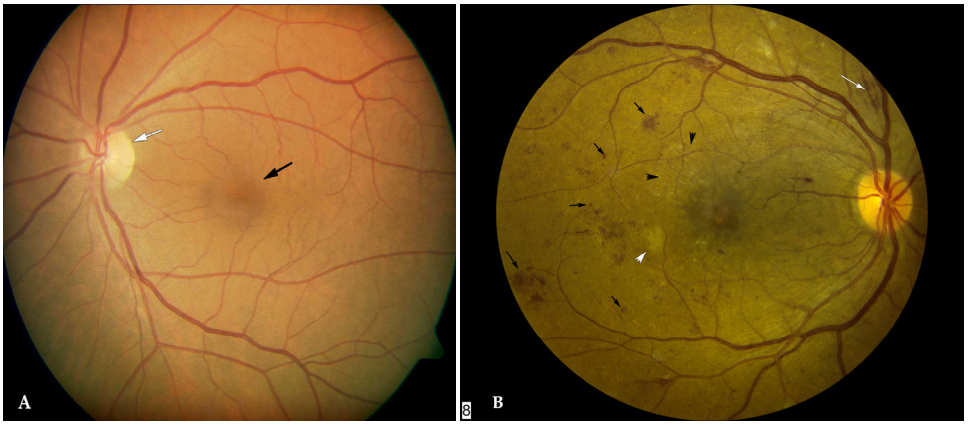

Fig. 3. A: Color photograph of normal fundus (left eye) normal optic disc (white arrow) and macula (black arrow). B: Color photograph (right eye) showing non-proliferative diabetic retinopathy and macular edema. Findings include; dot and blot hemorrhages (black arrows), flame shaped hemorrhages (white arrow), and cotton wool spot (white arrowhead) which represent nerve fiber layer infarcts. Hard exudates (black arrowheads) are lipid filled macrophages and in this photograph are radially distributed around the central macula.

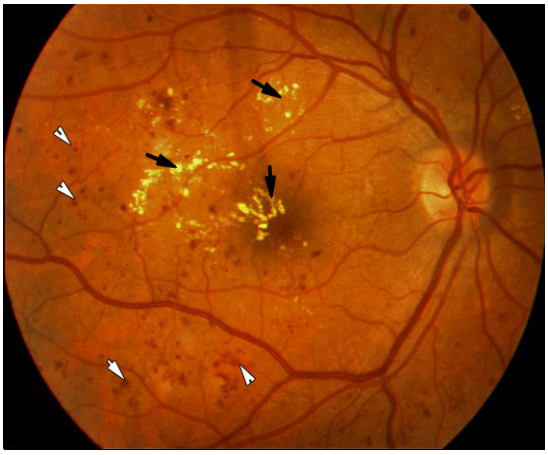

Fig. 4. Color photograph of fundus with moderate non-proliferative diabetic retinopathy with macular edema. Multiple dot and blot retinal hemorrhages are seen (white arrowheads) and hard exudates (black arrows).

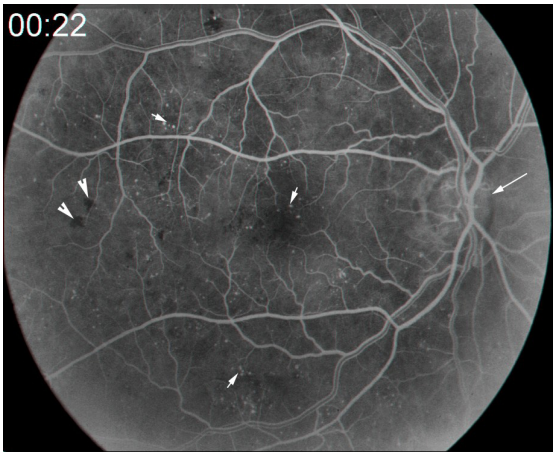

Fig. 5. Fluorescein angiography of non proliferative diabetic retinopathyof the right eye. Sodium fluorescein is injected into the systemic circulation, and an angiogram is obtained by photographing the fluorescence emitted after illumination of the retina. In NPDR the FA shows microaneurysms filled with dye (small white arrows). Hemorrhages appear as black dots because the transmission of fluorescence from below the hemorrhage is blocked (white arrowheads) . The optic disc marked with long white arrow.

Stage of NPDR	Clinical Features	Progression Risk
Mild NPDR	Few microaneurysms	5% progress to PDR within 1 year
Moderate NPDR	Microaneurysms and other microvascular lesions	12-16% progress to PDR within 1 year
Severe NPDR (Meets 1 of 3 criteria)	• Extensive intraretinal hemorrhages and microaneurysms in all four quadrants • Venous beading in two or more quadrants • One IRMA	52% progress to PDR within 1 year 15% progress to high risk PDR within 1 year
Very severe NPDR	Any two of the features of severe NPDR	45% progress to high risk PDR within 1 year

Table 1. Clinical classification of non-proliferative diabetic retinopathy

7.4.2 Proliferative diabetic retinopathy (PDR)

Diabetic retinopathy advances to the proliferative stage when new vessels (neovascularizations) are formed which grow up from the retina towards the vitreous cavity. The development of these pathological blood vessels is induced by pro-angiogenic factors released as a result of the severe retinal ischemia caused by the progression of diabetic retinal microvascular disease. Neovascularizations can be identified clinically as a jumble of disorganized, fine vessels emanating from the organized retinal vessel architecture (figure 6). Fluorescein angiography is also very effective at identifying

neovascular lesions as the new vessels are porous and leak fluorescent dye into the vitreous cavity.

The new vessels in PDR evolve in three stages. Initially, the fine new vessels grow with minimal fibrous tissue. Then the new vessels increase in gauge and length with an increased fibrous component. Finally, the vessels regress and the residual fibrovascular tissue along the posterior surface of the vitreous body contracts.

Retinal neovascularizations (NV) are divided into two subtypes based on their relative risk of causing severe visual loss as demonstrated by the Diabetic Retinopathy Study (DRS). Vascular proliferations on or near the optic disc are termed NV-disc (NVD) and proliferations elsewhere are termed NV-elsewhere (NVE) (figures 6 - 8). The presence of NVD carries the higher risk of severe visual loss and requires more urgent treatment (DRS research group, 1979, 1981).

PDR is graded from early to high risk based on the risk of severe visual loss as determined by the extent of the neovascular proliferations. The DRS (DRS research group, 1979, 1981) defined high risk PDR as the presence of either: NVD with a vitreous hemorrhage, NVD larger than a quarter disc area without vitreous hemorrhage or NVE larger than half disc area with vitreous hemorrhage. Without treatment, patients with early PDR have 50% risk of developing high risk PDR in 1 year and those with high risk PDR have a 25% risk of severe visual loss within 2 years. Treatment of PDR involves extensive peripheral laser ablation of the retina and is discussed in section 7.5.2.3.

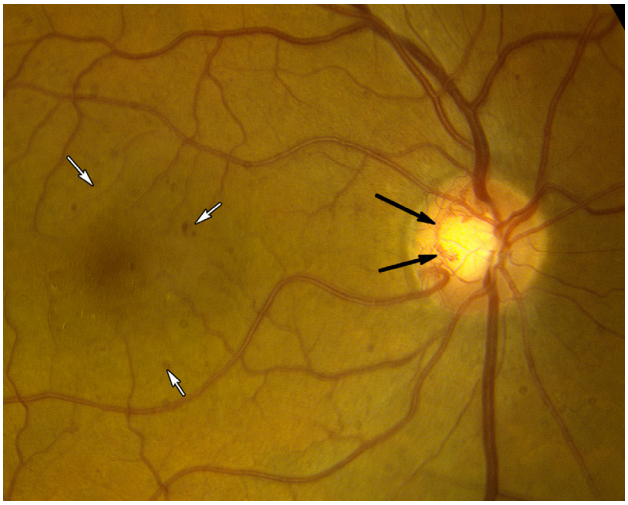

Fig. 6. Proliferative Diabetic Retinopathy with Neovascularization of the Optic Disc (NVD). Vascular proliferations on or near the optic disc are termed NV-disc (NVD).This Color photograph shows fine jumbled vessels (black arrows) typical of NVD. Multiple dot and blot retinal hemorrhages are seen (white arrows).

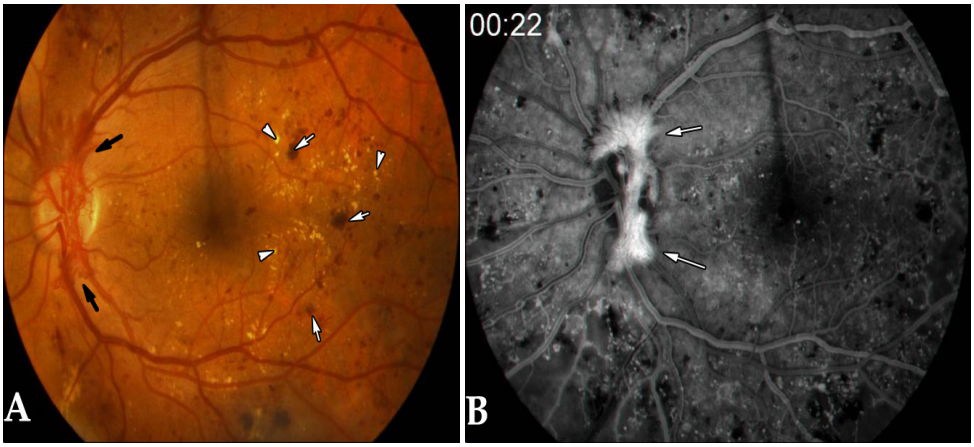

Fig. 7. High Risk Proliferative Diabetic Retinopathy with Large NVD. PDR is graded from early to high risk based on the risk of severe visual loss as determined by the extent of the neovascular proliferation. A: Color photograph showing a large neovascularization of the optic disc (black arrows) this NVD is larger than a quarter disc area therefore consistent with high risk PDR. Retinal hemorrhages (white arrows) and hard exudates (white arrowheads) are also seen. B: Fluorescein angiography in the same patient demonstrating hyper-fluorescence due to dye leakage from the disc neovascularization (white arrows).

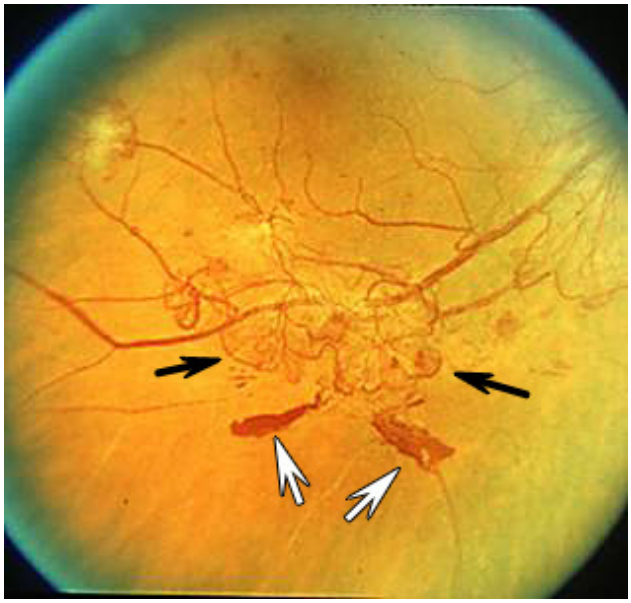

Fig. 8. High Risk Proliferative Diabetic Retinopathy with large NVE. This neovascular lesion located away from the optic disc is known as a Neovascularization-Elsewhere (NVE). This large NVE (black arrow) is associated with a small hemorrhage (white arrow).

The most frequent complication of PDR is vitreous hemorrhage (figure 9) caused by rupture in the fragile neovascular vessels. The initial complaint is often of black dots partially obscuring vision and can evolve to severe visual loss over a period of hours to days as the eye fills with blood.

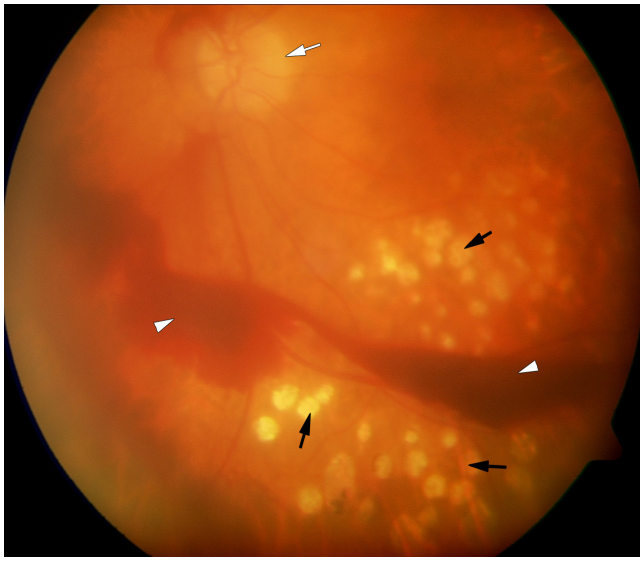

Fig. 9. Vitreous Hemorrhage. Vitreous hemorrhage is the most frequent complication of PDR. It is caused by rupture of neovascular vessels. This color figure shows partial vitreous hemorrhage causing general haze. Dense blood in the vitreous cavity is seen (white arrowheads). This hemorrhage occurred in eye after the initiation of pan-retinal photocoagulation as a treatment for PDR. Multiple white-yellow laser burns are seen (black arrows). This treatment is discussed in section 7.5.2.3. The optic disc is also seen (white arrow).

Another cause of severe vision loss in PDR is traction retinal detachment. This detachment occurs when the neovascular tissue connecting the retinal surface to the vitreous contracts causing a separation between the surface and deep retinal layers (figure 10). If this occurs in the center of the macula severe vision loss can result.

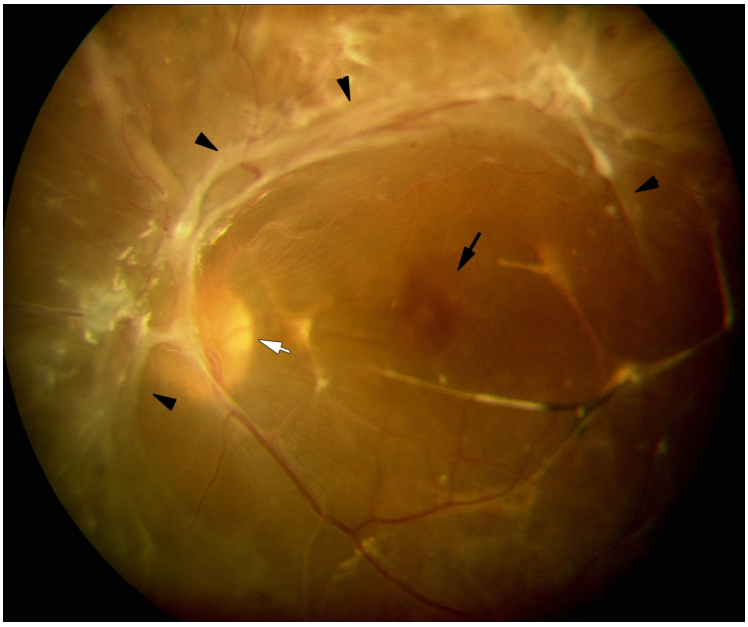

Fig. 10. Traction Retinal Detachment. This detachment is one of the serious complications in PDR and may cause severe visual loss. It occurs when the neovascular tissue connecting the retinal surface to the vitreous contracts causing a separation between the surface and deep retinal layers. This color fundus photograph shows elevated and detached retina (black arrowheads) involving the optic disc area (white arrow). The retina at the center of the macula (black arrow) is not detached.

A third serious complication of PDR occurs when proliferative and pro-angiogenic factors released from the ischemic retina induce the propagation of new vessels on the iris (figure 1). These vessels can block the normal outflow of fluid from the eye causing often severely increased ocular pressure. This complication is known as neovascular glaucoma and is discussed further previously in section 5.

Visual acuity in the absence of macular disease is often good in PDR until a complication occurs, most commonly vitreous hemorrhage. This sudden transition from good vision to near blindness can be traumatic for patients who were unaware of the severity of their diabetic eye disease.

7.4.3 Diabetic macular edema

Diabetic macular edema (DME) is responsible for most of the moderate visual loss in retinopathy patients. The vision loss is often mild at first, but without effective treatment it can progress and patients can lose the ability to perform activities of daily

living such as reading and driving. Diabetic macular edema is assessed separately from the stage of retinopathy (NPDR/PDR) and it can manifest along a different and independent course.

The edema evolves when damage to the macular capillary bed causes a breakdown of the blood-retina barrier. This results in increased retinal vascular permeability and to the accumulation of fluid in the macula. Macular edema may be 'focal' and emanate from a small cluster of leaky vessels, or 'diffuse' and involve the entire macula without a clear point of origin. Clinical examination can reveal rings of hard exudates (lipid filled macrophages) which delineate the area of focal leakage (figures 3 & 4). These subtypes are often differentiated by angiography which demonstrates areas of focal leakage from specific capillary lesions and microaneurysms in focal edema. In diffuse macular edema, angiography reveals widespread leakage with no definitive point of origin from extensive breakdown of the blood-retinal barrier (Regillo et al., 2010).

Treatment decisions are based on the clinical examination in DME. Intervention is recommended only when the retinal edema involves or threatens the center of the macula. In all other cases, close follow-up alone is indicated (ETDRS group, 1995).

Optical Coherence Tomography (OCT) is a useful ancillary imaging technique in DME. Recent technological advances in OCT technology have provided ophthalmologists with high-resolution images of the retina in cross-sectional slices. Aside from demonstrating areas of retinal thickening and intra-retinal fluid (figure 11), OCT obtains quantitative measurements of central retinal thickness that are important for close monitoring and follow-up of macular edema. Serial OCT examinations are often used as a non-invasive and accurate method analyzing treatment response in DME patients (Cheung et al., 2008).

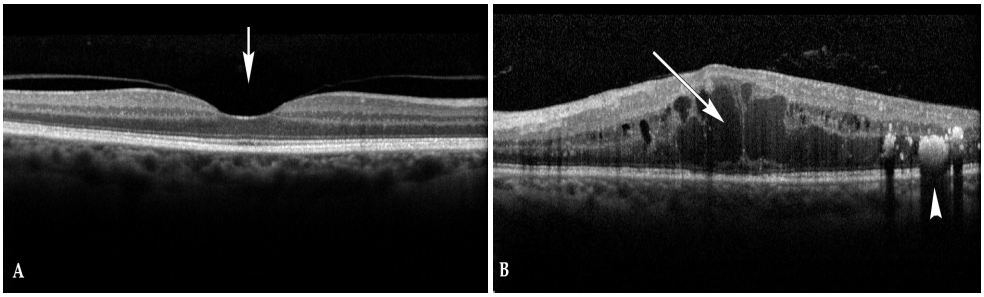

Fig. 11. Optical Coherence Tomography (OCT) of the Macula. A: OCT scan demonstrating the normal anatomic indentation in the central macula in a healthy eye (white arrow). B: Diabetic macular edema: There is loss of central macular indentation due to retinal cysts (white arrow). Hard exudates are also seen (white arrowhead).

7.4.4 Diabetic macular ischemia

Macular ischemia is a devastating complication of diabetic retinopathy. It is caused by extensive loss of retinal capillary perfusion in the macula. Clinical exam often reveals microaneurysms clustering at the margins of the non-perfused retina. Angiography can demonstrate the presence and extent of the area with capillary non-perfusion. This entity is generally associated with significantly decreased vision (Regillo et al., 2010).

7.5 Treatment and prevention of diabetic retinopathy

The main goal of treatment of diabetic retinopathy is preventing complications which can lead to vision loss. Treatment should include both ocular therapy and systemic medical intervention.

7.5.1 Medical treatment

Hyperglycemia, hypertension and hyperlipidemia are known risk factors for the development and progression of diabetic retinopathy. Treating and controlling these factors is crucial to preventing and limiting disease progression.

The Diabetes Control and Complications Trial (DCCT group, 1995) showed that intensive glycemic control reduced both the risk of developing retinopathy and the rate of progression of existing retinopathy. Intensive glycemic control reduced the risk for progression to severe NPDR and PDR, the incidence of diabetic macular edema and the need for laser treatments. Every percent reduction in hemoglobin A_{1C} lowers the risk of retinopathy development by 30-40%.

Several other systemic therapies have been investigated and found to reduce the risk of retinopathy progression including Angiotensin Converting Enzyme Inhibitors, Protein Kinase C Inhibitors and inhibitors of Advanced Glycosylation End-products formation (Cheung et al., 2008).

7.5.2 Ocular treatment

Ocular therapy in diabetic retinopathy includes panretinal or focal laser photocoagulation, intravitreal injections of either steroids or inhibitors of Vascular Endothelial Growth Factor (VEGF), surgery or a combination of the aforementioned treatments. The suitable treatment regimen must be tailored individually for each patient and is based on clinical status of the patient (ocular and systemic), previous treatments and data from several reports and ongoing studies.

7.5.2.1 NPDR

Visual acuity is not usually affected in NPDR unless there is damage to the macula in the form of macular edema or ischemia. Ocular treatment at this stage is definitively indicated only if there is evidence of macular disease. In patients with very severe NPDR who are at high risk for progression to PDR, laser treatment can be considered if the patient is not considered a suitable candidate for close follow-up. In such cases the recommended treatment is Pan- Retinal laser Photocoagulation (PRP) which will be discussed in section 7.5.2.3 (ETDRS group, 1995).

7.5.2.2 Diabetic macular edema (DME)

Treatment options for DME include focal laser photocoagulation, intravitreal injections of either steroids or anti-VEGF compounds and surgery.

7.5.2.2.1 Focal laser

The mainstay of DME treatment is focal laser photocoagulation. Focal laser treatment for DME involves the application of discrete laser burns to areas of leakage in the macula. The treatment is not painful and can be repeated up to every 4 months if edema persists. Treatment criteria are based on the ETDRS recommendations (ETDRS group, 1995) which showed that eyes with macular edema involving or threatening the central macula, defined

as clinically significant macular edema (CSME), benefited from focal laser treatment. Focal laser treatment reduced the risk of moderate visual loss (loss of three lines of vision) by 50% over two years, increased the chance of improved vision and reduced central macular thickness compared to no treatment.
In eyes with macular edema that does not meet the criteria for CSME, laser treatment is not indicated. Close follow-up is recommended to determine the progression of the macular edema. Unfortunately, when the macular edema is associated with macular ischemia from a loss of macular capillary perfusion, the ETDRS showed a lesser beneficial effect for focal laser (ETDRS Group, 1995).
Side effects of focal laser photocoagulation include: paracentral visual field loss, transiently increased macular edema with decreased visual acuity and choroidal neovascularization (Regillo et al, 2010).

7.5.2.2.2 Steroid injections

Inflammatory factors play an important role in the development of diabetic retinopathy and macular edema (see section 7.3). For this reason it has long been thought that ocular steroid injections may be beneficial in DME treatment. Several trials have shown modestly improved visual acuity and central macular thickness after injection of intravitreal Triamcinolone (Grover at al., 2008; Yilmaz et al., 2009). A few recent trials on long acting steroid implants, such as Fluocinolone Acetate or Dexamethasone, have also reported short term visual acuity improvements (Grover et al., 2009).
The Diabetic Retinopathy Clinical Research Network (DRCR network, 2008) compared intravitreal injection of Triamcinolone to focal laser treatment in eyes with DME. There was no difference in visual acuity between the two groups after 1 year, and at 2 years eyes treated with laser had better vision. However, complications of intravitreal steroids, including elevated ocular pressure and increased cataract progression limit the usefulness of these drugs in DME. Intravitreal sterioid injections may be considered in patients who have previously undergone cataract surgery and in cases where the macular edema is refractory to focal laser. In these cases the injections may be given either alone or as an adjunct to laser treatment (Gillies et al., 2006; Maia et al., 2009).

7.5.2.2.3 Anti-vascular endothelial growth factor (VEGF) compounds

Vascular Endothelial Growth Factor (VEGF) is a major cause of the increased retinal vascular permeability which causes macular edema (Stirban et al., 2008). Several VEGF inhibitors have been investigated as treatments for DME with a beneficial effect on visual acuity and central macular thickness.
Injection of intravitreal Pegaptanib, a pegylated aptamer that inhibits one isoform of VEGF, was found to be better than sham injections in improving in visual acuity and decreasing the need for focal laser treatment, in the Macugen Diabetic Retinopathy Study group (Cunningham et al., 2005).
The injection of monoclonal antibodies that block all isoforms of VEGF has also been investigated as a treatment for DME. Several studies have shown a beneficial effect on visual acuity in eyes treated with Bevacizumab (trade name Avastin), a recombinant full length humanized antibody to VEGF (Nicholson & Shachat, 2010). Based on data from multiple studies (Nicholson & Shachat, 2010) repeated doses of Bevacizumab increase its average positive effect. The optimal timing for repeat dosing is unclear, but is probably between 3 to 12 weeks, with maximal effect with a 3 to 6 weeks interval between treatments.

The Bevacizumab Or Laser Treatment study (Michaelidis et al., BOLT study, 2010) compared 6-weekly bevacizumab injections to focal laser treatment in DME. One year post-randomization, eyes treated with bevacizumab had significantly better vision by over one line of acuity and less macular edema compared to eyes treated with focal laser. Bevacizumab treated eyes also had fivefold greater odds gaining at least 2 lines of vision.
Another promising drug that targets VEGF is Ranibizumab (trade name Lucentis). This a recombinant, humanized antibody fragment binds and inhibits all isoforms of VEGF. A recent study compared four treatment options; monthly injections of Ranibizumab combined with focal laser, monthly Ranibizumab alone with the option for rescue focal laser treatment, focal laser treatment alone and focal laser combined with intravitreal injections of Triamcinolone (DRCR network 2010a). After 1 year, eyes that received intravitreal injections of Ranibizumab, either combined with laser or with the option for rescue laser had better visual acuity compared with the other treatment groups. On average, eyes receiving Ranibizumab gained 1 line in visual acuity after 1 year. Half of Ranibizumab treated patients gained more than 2 lines in visual acuity, and 30% gained 3 lines or more. Two years results showed a similar positive treatment effect in DME with Ranibizumab injections.
The injection of anti-VEGF agents to the vitreous is both effective and safe. Adverse ocular effects include: cataract formation, retinal detachment, vitreous hemorrhage and infection. Potential systemic adverse effects include: hypertension, stroke, and myocardial infarction but these are very uncommon (Cheung et al., 2010; Nicholson & Shachat, 2010).

7.5.2.2.4 Surgical intervention

DME can also be treated surgically by performing a vitrectomy. This option is used sparingly because of the utility of both laser treatments and intravitreal injections in controlling the disease. Surgery is indicated in cases refractory to other treatments or when there is mechanical traction on the macula from vitreo-retinal adhesions. In such cases edema resolution can often not be obtained without resorting to intraocular surgery where the traction can be definitively released (Kaiser et al., 2001; DRCR network, 2010b).

7.5.2.3 Proliferative diabetic retinopathy (PDR) treatment

7.5.2.3.1 Panretinal photocoagulation (PRP)

The goal of treatment in PDR is to prevent complications and lower the risk of severe vision loss. The mainstay of treatment for PDR is laser ablation of the peripheral retina. In this treatment, known as panretinal photocoagulation (PRP), laser burns are placed over the entire retina, sparing only the central macula (figure 12). PRP promotes the regression and arrest of progression of retinal neovascularizations by destroying ischemic retinal tissue and reducing ischemia driven VEGF production (Cheung et al., 2010; Regillo et al., 2010).
The Diabetic Retinopathy Study (DRS) evaluated efficacy of PRP treatment in eyes with advanced NPDR or PDR (DRS Group, 1981). The DRS study recommended prompt treatment in eyes with high risk PDR (defined in section 7.4.2), because these eyes had the highest risk for severe visual loss. PRP treatment in these patients reduced the risk of severe visual loss by 50% over 5 years. The ETDRS study found that PRP treatment in eyes with early PDR reduced the risk of progression to high risk PDR by 50%, and significantly reduced the risk of severe visual loss (ETDRS Group, 1995). Based on these results, PRP treatment should be considered in eyes with any stage PDR especially if there is poor metabolic control, a non compliant patient or difficulty in maintaining close follow-up.

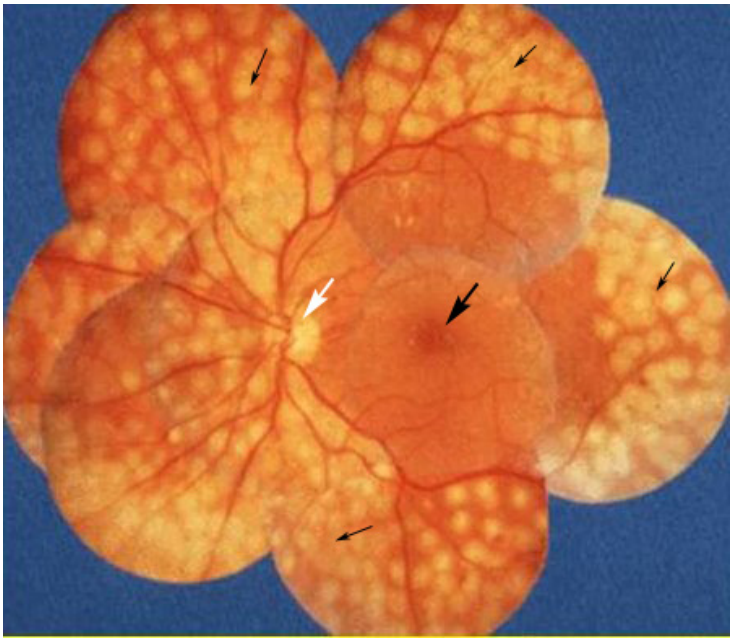

Fig. 12. Pan-Retinal Photocoagulation. The mainstay of treatment for PDR is laser ablation of the peripheral retina. In this treatment, known as panretinal photocoagulation (PRP), laser burns are placed over the entire retina (small black arrows), sparing only the central macula (large black arrow) and the optic disc (white arrow).

Full PRP treatment as recommended by the DRS and the ETDRS includes as many as 4000 laser burns. PRP can be painful and is often performed over several sessions. After the initial treatment course, additional therapy can be applied if there is persistent neovascularisation. After treatment, proliferative retinal tissue may regress and contract causing a vitreous hemorrhage or a traction retinal detachment from contracture of fibrovascular tissue. Side effects of PRP treatment also include; decreased in night vision, decreased color vision and loss of peripheral vision. These side effects can be reduced by spreading out the treatment sessions and by using less energy at each session (Regillo et al. 2010).
When PDR presents with macular edema, PRP treatment may initially increase the amount of the edema (ETDRS Group, 1991). In such case it is recommended to treat the macular edema with either focal laser or an intravitreal injection before initiating PRP (Silva et al., 2009, Mirshani et al., 2008).

7.5.2.3.2 Vitreous hemorrhage

In patients with new onset vitreous hemorrhages, laser PRP treatment is recommended if visualization of the retina is adequate. A severe, dense, non-clearing vitreous hemorrhage is an indication for vitrectomy surgery. The Diabetic Retinopathy Vitrectomy Study (DRVS Group, 1985) recommended surgery within 1 to 6 months of vitreous hemorrhage onset in type 1 diabetes patients with a non-clearing vitreous hemorrhage. Early vitrectomy improved visual acuity outcomes compared with waiting up to a year for spontaneous resolution before resorting to surgery.

Recent advances in vitreoretinal surgery, including smaller gauge instruments and the ability to perform laser ablation during surgery, have changed treatment recommendations. If a patient with a vitreous hemorrhage has not previously undergone PRP, vitrectomy is recommended when a dense vitreous hemorrhage persists beyond one to three months. Patients with vitreous hemorrhage that have preexisting complete PRP may undergo a longer observation period (Regillo et al., 2010).

Several studies have evaluated the efficacy of intravitreal anti-VEGF injections in patients with PDR (Nicholson & Shachat, 2010). Intravitreal Bevacizumab as adjunctive therapy with PRP was found to decrease leakage area from neovascularizations, improve visual acuity outcomes and reduce macular edema compared with PRP alone. In eyes with PDR and a dense vitreous hemorrhage preventing full PRP treatment, a Bevacizumab injection has been shown to aid significantly in clearing the hemorrhage (Moradian et al., 2008).

Bevacizumab has also been shown to enhance retinal surgery in patients with PDR. A single Bevacizumab injection given 1-2 weeks before vitrectomy for vitreous hemorrhage, results in decreased bleeding during surgery, decreased operating time and less post operative vitreous hemorrhage as compared to vitrectomy alone (Nicholson & Shachat, 2010; Ahmadieh et al.,2009).

7.5.2.3.3 Traction retinal detachment

Traction retinal detachment from the contraction of the neovascular tissue connecting the retinal surface to the vitreous is another serious complication of PDR. However, traction detachments which do not involve the macula can remain stable for years. Vitrectomy surgery is indicated only when the traction retinal detachment involves or threatens the central macula or if a retinal tear develops (Regillo et al. 2010).

7.5.2.3.4 Neovascular glaucoma

A third serious complication of PDR occurs when high levels of VEGF in the retina induce the development of new vessels on the iris. These vessels threaten to block the outflow of aqueous fluid from the eye and raise ocular pressure. The treatment of patients with neovascularisation of the iris involves both the minimization of retinal ischemia and the aggressive reduction of intraocular pressure if elevated. Retinal ischemia is treated with aggressive and extensive ablation of peripheral retinal tissue with PRP regardless of the stage of PDR. Injection of intravitreal anti-VEGF agents as adjunctive therapy to PRP can induce a rapid reduction or resolution of neovascularisation of the iris (Ahmadieh et al., 2009; Wasik et al., 2009). However, these injections should be seen as an adjunct to full PRP which remains the definitive treatment and not as a viable replacement.

Elevated ocular pressure in neovascular glaucoma is treated initially with topical medications. Often multiple drops are required to reduce pressure to below the target level of approximately 20 millimeters of mercury. In advanced cases, topical treatment alone may not be sufficient and systemic treatment with carbonic anhydrase inhibitors such as Acetazolamide may be considered. Common side effects of Acetazolamide include numbness and tingling in the fingers and toes, and taste alterations. Acetazolamide also increases the risk of dehydration and metabolic acidosis. Serial electrolyte and kidney function tests are recommended in all patients receiving this medication.

In refractory cases Cyclodestructive procedures are required if medical therapy fails to provide symptomatic relief. With cyclocryotherapy, the IOP-lowering effect is achieved by destroying secretory ciliary epithelium and/or reducing blood flow to the ciliary body. It is indicated as a last resort only if relief of pain is the main goal.

7.6 Special considerations

7.6.1 Diabetic retinopathy in pregnancy

In women with preexisting diabetes, pregnancy is considered an independent risk factor for the development and progression of diabetic retinopathy (Shultz et al., 2005). Gestational diabetes, in absence of preexisting diabetes does not show a similar association with diabetic retinopathy. Most of the progression of diabetic retinopathy in pregnancy occurs by the end of the second trimester. Although regression of retinopathy usually occurs postpartum, there is still an increased risk for progression during the first year postpartum (Shultz et al., 2005). Risk factors for the development and progression of diabetic retinopathy in pregnancy include longer duration of diabetes before conception, rapid normalization of hemoglobin A_{1C} at the beginning of pregnancy, poor glycemic control during pregnancy, diabetic nephropathy, high blood pressure and preeclampsia (Shultz et al., 2005; Vestgaard et al., 2010).

Severity of diabetic retinopathy before or at beginning of pregnancy is also a strong predictor of progression of retinopathy during and after pregnancy. The Diabetes in Early Pregnancy Study (Chew et al., 1995) showed that 10.3% of women without diabetic retinopathy and 18.8% with mild NPDR experienced retinopathy progression during pregnancy, and 6.3% of women with mild NPDR progressed to PDR. In women with moderate NPDR, 54.8% suffered retinopathy progression and 29% developed PDR. Overall, progression to sight threatening diabetic retinopathy, including macular edema and PDR, occurs in 6% of pregnant diabetic women (Vestgaard et al., 2010).

Progression of retinopathy during pregnancy is probably related to the hypervolemic and hyper-coagulable states in pregnancy, as well as elevated pro-inflammatory and angiogenic factor levels. This results in capillary occlusion and leakage aggravating diabetic retinopathy mechanisms (Shultz et al., 2005; Kastelan et al., 2010). Ideally, good glycemic control and full treatment of pre-existing diabetic retinopathy complications should be attained before conception.

All diabetic women who plan pregnancy should be referred by their treating physician to an ophthalmologist. The recommended follow-up of pregnant women with type 1 diabetes includes an ophthalmologic exam at the beginning of pregnancy and during the first trimester. Subsequent follow-up depends on the stage of diabetic retinopathy found on the initial examinations. In women with no retinopathy or very mild NPDR, an ophthalmologic exam is indicated when there are visual complaints. In moderate NPDR an exam should be done at least once during the second trimester and every 4-6 weeks during the third trimester. In severe NPDR and PDR, close follow-up is needed, and an exam should be done every 4-6 weeks, from the beginning of the second trimester.

Treatment of diabetic retinopathy during pregnancy includes maximal control of both glucose levels and blood pressure (Vestgaard et al., 2010). Ocular therapy such as PRP should definitely be performed for PDR and be strongly considered in cases of severe NPDR. Disease progression can be very fast in pregnancy and waiting for PDR to clearly

develop may result in severe complications that necessitate invasive surgery. Ocular therapy for PDR and macular edema during pregnancy can include PRP, focal laser and intravitreal injections of Triamcinolone. Although there is not much data on the safety of intravitreal injections of anti-VEGF agents during pregnancy, the literature includes some reports on the safe and effective use of Bevacizumab (Tarantola et al., 2010).

7.6.2 Cataract surgery in patients with diabetic retinopathy

Cataract is a major factor which compromises vision in diabetic patients. While diabetics may benefit from cataract extraction, a controversy exists in the ophthalmic community as to whether cataract surgery potentiates diabetic retinopathy progression. Several studies have reported worsening of diabetic retinopathy and macular edema after surgery (Pollack et al., 1991; Hauser et al., 2004; Jaffe et al., 1992, Hayashi et al., 2009). Progression was seen during the first year after surgery and was highest in the first 3 months post-operatively. A review of several other studies, especially in the cataract surgery era using the smaller incision phacoemulsification technique, showed no significant progression of diabetic retinopathy and macular edema after surgery (Rashid & Young, 2010; Shah & Chen, 2010). Overall, diabetics with cataracts benefit from surgery, and improved visual acuity is reported in 92-94% of patients (Rashid & Young, 2010). The combined evidence suggests that in patients with low risk or absent diabetic retinopathy and no clinically significant macular edema at the time of surgery, there is little increased risk of retinopathy progression. However, in patients with severe NPDR, PDR or significant macular edema, cataract surgery carries an increased risk for retinopathy progression and a worse visual acuity outcome.

Recent studies have shown a potential benefit using intravitreal injection of Bevacizumab at the end of cataract surgery (Cheema et al., 2009; Chen et al., 2009; Nicholson & Shachat, 2010) especially in cases with poorly controlled or refractory macular edema and diabetic retinopathy before surgery. Patients who received intravitreal Bevacizumab enjoyed better outcomes in terms of visual acuity, macular thickness and retinopathy progression.

A thorough evaluation of patients with diabetes is warranted before cataract surgery. Patients who have severe NPDR or PDR should be considered for PRP treatment prior to cataract removal (Chew et al., 1999). Patients with clinically significant macular edema should undergo treatment, such as focal laser or intravitreal injection of anti- VEGF agents pre-operatively. Ideally, surgery should be delayed until stabilization of retinopathy and macular edema is achieved. In refractory cases, adjunctive therapy with an anti-VEGF agent at the end of cataract surgery should be considered. Close post-operative follow-up with an ophthalmologist is highly recommended in all patients with preexisting diabetic retinopathy.

8. Schedule for ophthalmologic examinations

Regular ocular examination can detect early ocular disease such as cataracts and glaucoma as well as retinopathy. Diabetic retinopathy in type 1 diabetes is rare during the first 5 years after diagnosis, so the baseline ophthalmologic examination could be extended to 5 years after diagnosis if blood glucose has been well controlled. In children with pre-pubertal diabetes, the baseline examination should be done at puberty (Raab et al. 2010).

The timing and frequency of follow-up ocular examinations depends on individual patient's status. In high risk patients with long term diabetes and poor systemic risk factor control annual examinations should be performed even in the absence of retinopathy. In patients with known retinopathy, the examination schedule is based on the degree of retinopathy, and on the patient's compliance and adherence to regular follow-up. In more advances stages such as PDR and when macular edema is present, more frequent and careful follow-up is suggested. (Regillo et al., 2010). Table 2 shows the recommended schedule for follow–up.

Retinopathy Stage	**Follow-up Schedule**
Normal or rare microaneurysms	Annually
Mild NPDR	Every 9 months
Moderate NPDR	Every 6 months
Severe NPDR	Every 2-4 months
Clinically significant macular edema	Every 2-4 months
PDR	Every 2-3 months (careful follow-up)

Table 2. Suggested time table for follow-up in diabetic retinopathy (modified from the Preferred Practice Patterns committee, retina panel, diabetic retinopathy, American Academy of Ophthalmology, 2003, as cited in Regillo et al., 2010).

9. Summary

Management of type 1 diabetes involves close cooperation between the treating primary physician and the many specialists who help manage the complications of this disease. Recent advances, including intraocular anti-VEGF injections, have added important new tools which minimize vision loss in diabetic eye disease. Proactive, interdisciplinary coordination of treatment and timely referrals can aid in the minimization of visually threatening complications, significantly enhancing patient quality of life.

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Part 4

Treatment

15

Perspectives of Cell Therapy in Type 1 Diabetes

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1. Introduction

Type 1 diabetes is an autoimmune disease leading to the destruction of pancreatic β cells. The reduction of β cell mass results in insulin deficiency that leads to a failure of glucose homeostasis with increased levels of glucose in blood. Hyperglycemia which *per se* is detrimental for the organism and may be life-threatening, may in the long term associate with chronic complications involving blood vessels and nerves. The gold standard treatment for diabetes patients aimed to reach a tight control of glycemia, relies on intensive insulin therapy based on multiple daily injections or continuous subcutaneous infusion of insulin. A tight control of glycemia reached with such regimens was shown to significantly reduce the incidence of microvascular complications in respect to the conventional insulin therapy. Nevertheless, to reach an optimal control of blood levels may prove to be difficult when compared to the physiological condition where this is guaranteed by the pancreatic β cells (Suckale, 2008). Therefore, preservation of β cell mass could be an important therapeutic target to reduce microvascular complications and to improve the glycemia control (Gonez & Knight, 2010). It is generally accepted that the endocrine pancreas has some regenerative capabilities, although it is still debated which cells are involved in β cell turnover. In rodents the capability of adult pancreas to increase β cell mass has been documented in physiological conditions and after injury. The understanding of mechanisms involved in β cell turnover may therefore be relevant to design new therapeutic strategies aimed to maintain a β cell mass or to favour regeneration of β cells. These strategies however, should take to account the problem of recurrent autoimmunity that in type 1 diabetes not only impairs the original β cell mass, but may also limit the regenerative process. Indeed, autoimmune T lymphocytes may kill the β cells newly formed in response to injury (Fan & Rudert, 2009).

2. β cell regeneration: Contribution of stem/progenitor cells or replication of β cell?

The physiological turnover of the long-lasting endocrine cells of pancreas requires the generation of new cells even with a very slow kinetic.

The organ growth after birth requires a coordinated increase in the number of constitutive cells. Moreover, in the adult body most of the tissues and organs have the ability to replace the cells that die for physiological senescence or following limited injury. The source of newly formed cells may derive from resident stem/progenitor cells or from the ability of differentiated cells to re-entry into cell cycle and replicate themselves. The prevalence of these two mechanisms varies in different organs and tissues; however, they may result in the restoration of the original tissue conformation and function. We therefore should expect that also the endocrine pancreas retains the ability of regeneration in appropriate physiological conditions. The nature and the location of the cells involved in such processes, as well as the mechanisms involved in the activation of the regenerative processes, still remain largely unknown.

The concept of "stem cell" implies the ability of unlimited self renewal and of high multilineage differentiation potential into different types of mature cells. Therefore, stem cells play fundamental roles in organogenesis during embryonic development, and in the adult are responsible for the growth, homeostasis and repair of many tissues.

In the haematopoietic system, the intestine and the skin, tissues that require a high cell turnover, the stem cells are critical for maintaining their homeostasis. However, adult stem/progenitor cells are present in the majority of tissues and organs of mammalian organisms, including the central nervous system (Reynolds & Weiss, 1992), retina (Tropepe et al., 2000), skeletal muscle (Jackson et al., 1999), liver (Herrera et al., 2006) and kidney (Bussolati et al., 2005).

In tissues with a low rate of cell turnover, such as the kidney, the lung, the skeletal muscle and the liver, the resident stem cells may activate after injury and participate in tissue repair. Tissue resident stem cells preferentially generate differentiated cells of the tissue of origin, suggesting a relevant role in the postnatal growth of organs, in physiological turnover and in tissue repair. Tissue-resident adult stem cells are thought to co-localize with supporting cells within specific regions or specialized microenvironments in each tissue/organ, called stem cell niche (Jones & Wagers, 2008; L. Li et al., 2005; Moore & Lemischka, 2006). In bone marrow the haematopoietic stem cells (HSC) are located in the endosteal niche, associated with the osteoblasts of the inner surface of the cavities of trabecular bone that could provide factors able to regulate number and function of HSC (Mitsiadis et al., 2007) and in the perivascular area of sinusoids that could ensure homeostatic blood cell production and prompt responses to haematological stresses (Kiel et al., 2005). The other stem cells present in bone marrow are the mesenchymal stem cells (MSC), undifferentiated adult stem cells of mesodermal origin, which localize in perivascular areas in the bone marrow in close association with HSC (Shi & Gronthos, 2003) and that have the capacity to differentiate into cells of connective tissue lineages, including bone, fat, cartilage and muscle (Y. Jiang et al., 2002).

Other stem cell niches detected in mammals (da Silva Meirelles et al., 2008; L. Li & Xie, 2005) are the epithelial stem cell niche in skin that resides in the bulge area of the hair follicle beneath the sebaceous gland (Cotsarelis et al., 1990; Niemann & Watt, 2002; Sun et al., 1991), the intestinal stem cell niche located at the fourth or fifth position above the Paneth cells from the crypt bottom (Booth & Potten, 2000; He et al., 2004; Sancho et al., 2004) and the neural stem cell niche at the subventricular zone and the subgranular zone of the hippocampus where neural stem cells could reside and support neurogenesis in the adult brain (Doetsch et al., 1999; Temple, 2001).

A problem in the identification of tissue resident stem cells is that we do not know specific markers allowing tracing of pluripotent stem cells in various tissues. Therefore, resident stem cells are mainly defined by functional *in vitro* assays using cultured cells, and their *in vivo* exact localization and function remains elusive. Several studies suggest that the adult tissue resident stem cells belong to the MSC lineage (da Silva Meirelles et al., 2006). The minimal criteria to define human MSC established by *Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy* (da Silva Meirelles et al., 2008; Dominici et al., 2006), include cell positivity for CD105, CD73, and CD90 and negativity for CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR as well as osteo-, chondro-, and adipogenic-differentiation capabilities.

A perivascular location for MSC has been suggested, correlating these cells with pericytes providing an explanation for the presence of MSC virtually in all vascularized tissues (da Silva Meirelles et al., 2006). The perivascular zone may act as a MSC niche *in vivo*, where microenvironment factors may modulate their phenotype with transition to progenitor and mature cells. For many years the concept of niche has been associated with a hierarchical nature of stem cells that undergoing asymmetric division insure self-renewal and generation of a progeny with progressive loss of proliferative potential and gain of differentiated characteristics (Till et al., 1964). More recently, a continuum model of stem cell biology has been proposed (Colvin et al., 2004; Quesenberry et al., 2005). It has been postulated that the phenotype of stem cells is labile, it varies with position in the cell cycle and that it is reversible (Colvin et al., 2007). This cell-cycle reversibility is at the basis of the continuum model of stem cell biology, in which the phenotype of stem cells is reversibly changing during the cell cycle transit awaiting for a terminal-differentiating stimulus at a cycle-susceptible time. In this model the status of the cell cycle and the exposure to environmental factors play critical roles in the acquirement of different phenotypes by the same cell in different functional states (Quesenberry et al., 2007). Recently, Quesenberry and Aliotta proposed that the so-called niche consists in areas of influence which are continually adjusting to individual circumstances (Quesenberry & Aliotta, 2008). Based on these considerations, the refined regenerative system in mammalians does not need to position in each organ different stem cell types but it would be sufficient to maintain few undifferentiated cells with self-renewal capability that depending on the circumstances may vary their phenotype to replace the loss of differentiated cells. On the other hand, differentiated cells may re-acquire an undifferentiated phenotype and re-entry in to cell cycle first to restore the cell mass and subsequently to re-differentiate and restore functional integrity. In this context, the exchange of genetic information among cells by microvesicles in a defined environment plays a critical role in modulating plasticity of stem cells as well as the response of differentiated cells to injury (Deregibus et al., 2010).

Studies on β cell proliferation in humans are limited, but there is evidence that this process occurs at relatively high levels in the first 2 years of life declining thereafter with the possibility, at least in animals, of re-induction under conditions of insulin-resistance, such as pregnancy or obesity. (Meier et al., 2008). This suggests that β cells may retain an intrinsic capacity to replicate.

In the adult, endocrine pancreas β cells are considered to have a very low turnover. However, albeit quite slowly, β cells undergo senescence and should be continuously replaced by newly formed cells. By combining abdominal CT scans and morphometric analysis of human pancreatic tissue, Meier et al reported that the β cell mass expands by

several fold from birth to adulthood as result of an expansion in size of islets rather than an increase in number of islets (Meier et al., 2008). The increase in β cell number per islet mainly occurs in young children in coincidence with the growth of the organ size. Cnop et al provided evidence for a long lifespan and low turnover of human islet β cells estimated by mathematical modelling of lipofuscin accumulation (Cnop et al., 2010). Human β cells, unlike those of young rodents, are long-lived and in the adult human β cell population is mainly established in the first 20 years of life. Dor et al using a method for genetic lineage tracing to determine the contribution of stem cells to pancreatic β cell neogenesis showed that pre-existing β cells, rather than pluripotent stem cells, are the major source of new β cells during adult life and after pancreatectomy in mice (Dor et al., 2004). These results suggest that terminally differentiated β cells retain, at least in mice, a significant proliferative capacity *in vivo*. Nir et al used a transgenic mouse model to study the dynamics of β cell regeneration from a transiently induced diabetic state (Nir et al., 2007). Lineage tracing analysis in this model indicated that enhanced proliferation of surviving β cells played the major role in regeneration. These studies provided evidence that adult pancreatic β cells are formed by self duplication rather than stem cell differentiation (Dor et al., 2004; Nir et al., 2007). On the other hand there are studies suggesting that regenerated β cells derive from precursors located within pancreatic ducts in the proximity of islets (Juhl et al., 2010). The origin from these precursors has been demonstrated in rodent models of pancreatic damage (Bonner-Weir et al., 2004; Xu et al., 2008). Monitoring the expression of Neurogenin 3 (Ngn3), the earliest islet cell-specific transcription factor in embryonic development, Xu et al showed activation of β cell progenitors located in the ductal lining in injured adult mouse pancreas (Xu et al., 2008). They found that differentiation of the adult progenitors is Ngn3 dependent and generates all islet cell types, including glucose responsive β cells that proliferate, both *in situ* and when cultured in embryonic pancreas explants. This study suggests that multipotent progenitor cells present in the pancreas of adult mice can increase the functional β cell mass by differentiation and proliferation rather than by self-duplication of pre-existing β cells only. Li et al investigated whether after partial pancreatectomy in adult rats, pancreatic-duct cells serve as a source of regeneration by undergoing a dedifferentiation and redifferentiation (W.C. Li et al., 2010). The Authors detected after pancreatectomy an early loss by the mature ducts of the ductal differentiation marker Hnf6, followed by the transient formation of areas composed of proliferating ductules, called foci of regeneration. These ductules expressed markers of the embryonic pancreatic epithelium Pdx1, Tcf2 and Sox9 (W.C. Li et al., 2010). Since foci subsequently form new pancreatic lobes, it was suggested that these cells act as progenitors of the regenerating pancreas. Islets in foci initially resemble embryonic islets as they transiently expressed the endocrine-lineage-specific transcription factor Ngn3 and lacked of MafA expression and contained low percentage of β cells. The numbers of MafA(+) insulin(+) cells progressively increased with the maturation of foci (W.C. Li et al., 2010). Based on these observations, it was suggested that adult pancreatic duct cells may recapitulate aspects of embryonic pancreas in response to injury (W.C. Li et al., 2010). This mechanism of regeneration implicates the plasticity of the differentiated cells within the pancreas.

As schematized in figure 1, after injury β cells may be replaced by replication of β cells or from differentiation of stem cells (SC) localized within the islets or in exocrine pancreatic tissues (ductal and acinal cells). After extreme loss of β cell mass, glucagon producing α cells may transdifferentiate in β cells. A possible contribution to β cell neogenesis comes from

bone marrow derived stem cells of both haemopoietic (BM-derived HSC) and mesenchymal (BM-derived MSC) origin. These cells act by a paracrine mechanism releasing factors that favour tissue repair. Moreover, transdifferentiation of ductal and acinal cells may generate insulin secreting cells.

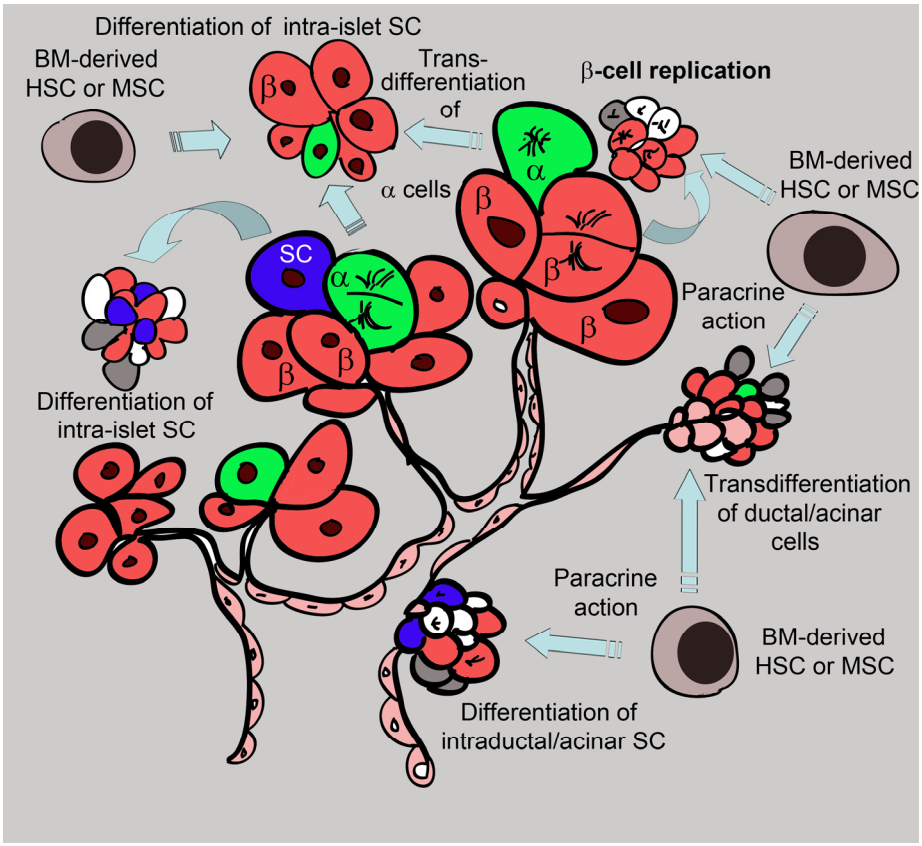

Fig. 1. Mechanisms potentially involved in β cell neogenesis.

The discrepancy in identification of cells that in the adult sustain β cell neogenesis, may result from the differential contribution in condition of physiological turnover and in condition of injury where the regenerative processes are accelerated and cells other than insulin producing β cells are involved to insure a reparative process. The study of Thorel et al showing the conversion of adult pancreatic α cells into β after extreme β cell loss stands in this line of interpretation (Thorel et al., 2010). In this study the Authors used *in vivo* genetic approaches to obtain near total β cell loss without autoimmunity combined with cell lineage tracing. For this purpose, they created a model of inducible, rapid β cell removal (>99%) by administration of diphtheria toxin (DT) in transgenic mice in which the DT receptor was expressed only in β cells (Thorel et al., 2010). In transgenic mice the systemic administration of DT permitted a specific cell ablation by apoptosis (Saito et al., 2001). In this model, β cell regeneration was monitored in combination with cell lineage tracing to investigate the

origin of newly formed β cells. The results obtained showed that the adult pancreas can generate new β cells after their near total loss, mainly by the spontaneous reprogramming of glucagon-secreting α cells (Saito et al., 2001). Therefore, β cell replication may account for maintaining homeostasis, whereas transdifferentiation of other cell types could be required after injury to replace the lost cells.
Summarizing these studies, one can envisage a scenario in which β cells may replicate themselves to maintain homeostasis (Dor et al., 2004), tissue specific precursors localized within pancreatic ductal cells generate α and β cells after pancreatic injury and glucagon secreting α cells can transdifferentiate into β cells to repair an extremely severe selective β cell loss. Translation of these experimental studies in humans may be difficult without the help of lineage tracing or of specific cellular markers.

3. β cell mass in type 1 and type 2 diabetes

Despite the evidence of some turnover in adult humans and the apparent capacity of endocrine pancreatic β cells to regenerate throughout life, patients with type 1 as well as type 2 diabetes have substantial deficit in β cell mass, approximately 99% in long-standing type 1 diabetes and 65% in long-standing type 2 diabetes. This loss of β cells implies that restoration of endogenous insulin secretion might be accomplished through replacement or regeneration of islet cells.
The β cell deficit in type 1 diabetes is related to the established autoimmune destruction of the target cells. However, several lines of research indicate that some β cell regeneration may occur in recent onset type 1 diabetic patients (Willcox et al., 2010) and even in patients with long-standing type 1 diabetes (Meier et al., 2005). The failure of β cell regeneration in type 1 diabetes may be related to the increased vulnerability of the newly forming β cells to apoptosis induced by inflammatory cytokines (Meier et al., 2006).
Other mechanisms, involved in all forms of diabetes, include the hyperglycemic induction of the nitric oxide synthase (NOS)-dependent mechanisms in islet microendothelium, with production of the vasoactive mediator nitric oxide (NO) (Suschek et al., 1994), endothelial cell loss and vasculature disruption (Zanone et al., 2008). NO is in fact increased in hyperglycemic conditions, and has an established direct islet cytotoxicity and potentially impairs insulin release (Steiner et al, 1997; Suschek et al., 1994; Kröncke et al., 1993). Islet microendothelial cells are also source of the proinflammatory cytokine IL-1β under hypeglycemic conditions, independently of any viral or immune-mediated process. IL-1β impairs insulin release in human islet, induces Fas expression enabling Fas-mediated apoptosis and IL-1β is thus implicated as a mediator of glucotoxicity (Maedler et al., 2001; Loweth et al., 1998). The metabolic mechanisms by which hyperglycemia initiates apoptosis in vascular endothelium are incompletely understood. These mechanisms include oxidative stress, increased intracellular Ca++, mitocondrial dysfunction, changes in intracellular fatty-acid metabolism, and impaired phosphorylation of the protein kinase Akt (Favaro et al., 2008). Akt signaling pathway plays a pivotal role in preventing apoptosis in a variety of settings (Datta et al., 1999), and, in particular, Akt activation is crucial for the ability of factors such as insulin, IGF-I and VEGF to inhibit apoptosis in cultured endothelium (Jung et al., 2000). Recent data highlight the Akt role also in insulin-mediated glucose transport and pancreatic β cell mass and function (Bernal-Mizrachi et al., 2004; Elghazi et al., 2006). As for type 2 diabetes, the progressive increase in glucose levels that characterizes its natural history has been claimed to be due to gradual reduction of function and mass of β cells, and

a significant reduction in β cell mass is clearly established in Type 2 diabetes (Donath & Halban, 2004; Weir & Bonner-Weir, 2004). The same mechanisms of glucotoxicity reported are involved, together with dyslipidaemia. In murine models of type 2 diabetes, short-term hyperglycemia has been shown to increase islet capillary blood pressure and perfusion, in a glucose dependent and reversible pathway, possibly mediated by a NOS–dependent mechanism (Bonner-Weir, 2004; Carlsson et al., 1998). However, with age, persistent hyperglycemia induces islet hypoperfusion. Inducible NOS (iNOS) increases the islet blood perfusion also in prediabetic low dose streptozotocin-treated mice, a model of type 1 diabetes (Carlsson et al., 2000). Increased islet capillary flow and pressure could, over time, contribute to the damage of the islet endothelium and thickening of the capillary wall, thus decreasing the islet perfusion. The hyperglycemia-induced NO production by the endothelium could also result cytotoxic to the islets and directly impair insulin release.
In Zucker diabetic fatty rats, a model of type 2 diabetes, it has been shown that changes in the islet vasculature play a key pathogenetic role in the development of diabetes (X. Li et al., 2006). In a biphasic pattern, an early vascular hyperplasia was followed by endothelial cell loss and vasculature disruption, in parallel to progressive islet failure.

4. Islet transplantation as strategy for β cell replacement

Allogenic pancreatic islet transplantation has become a suitable therapeutic option for the treatment of patients with unstable type I diabetes after the introduction of the Edmonton Protocol based on the optimization of islet isolation techniques and the development of a rapamycin-based glucocorticoid-free immunosuppressive regimen (Ricordi, 2003; Shapiro et al., 2000, 2003, 2006). However, after 5 years only 10% of the recipients were insulin independent (Ryan et al., 2005; Shapiro, 2006). In addition, although a sufficient islet mass can be obtained from good quality pancreata, to achieve insulin independence usually are needed islet preparations derived from multiple donors (Biancone & Ricordi, 2002). Therefore, this procedure is limited by the supply of cadaveric donors. Moreover, several factors may be responsible of the progressive dysfunction of transplanted islets. After an initial islet mass loss following the intraportal infusion, due to an inflammatory reaction, engraftment requires efficient islet revascularization by a chimeric vascular tree formed by host and recipient endothelial cells (Brissova et al., 2004). A poor vascular engraftment is one of the main causes of islet loss. Other factors that concur to islet loss include the exposure of islets to increased lipid levels, a side effect of immunosuppressive therapy based on mTOR inhibitors (Hafiz et al., 2005; Pileggi et al., 2006). Exposure to high-dose of calcineurin inhibitors (CNI) is recognized to induce direct β cell toxicity and functional impairment. The antiproliferative effects of mTOR inhibitors and CNI inhibit tissue remodelling and reduce β cell self-renewal (Nir et al., 2007). Moreover, the anti-angiogenic activity of rapamycin is a potential limitation of the current immunosuppressive protocols, that may be particularly detrimental in the early engraftment phase (Cantaluppi et al., 2006). Therefore, to overcome these problems it is necessary to improve the recovery and quality of islet cells from a single-donor pancreas and to develop strategies allowing the inhibition of inflammatory reactions, the improvement of islet vascularization and of islet engraftment using safer and less cytotoxic immunomodulatory approaches (Mineo et al., 2009; Pileggi et al., 2006; Ricordi, 2003). Recently, increased islet yields have been obtained by improving techniques of pancreas recovery and preservation and of islet isolation and purification (Pileggi et al., 2006;Ricordi, 2003). On the other hand, peritransplant interventions based on

combination therapies have been proposed to inhibit allo- and auto-immune response minimizing the side effects and favoring development of T regulatory cells (Treg) to maintain long term tolerance and to favor β cell regeneration. Targeting the costimulatory molecules involved in T-cell activation and/or adhesion molecules by immunomodulatory agents now available for clinical applications could be an option to reduce the side effects of immunosuppression and the islet toxicity and to achieve specific immune tolerance (Ricordi & Strom, 2004). Several experimental studies suggest that a combined islet transplant and cell therapy with bone marrow–derived cells, mesenchymal cells, Treg, and tolerogenic dendritic cells may modulate recipient immune response and increase the engraftment and long term survival of islets (Mineo et al., 2008). This possibility is supported by recent clinical trials demonstrating stable mixed haematopoietic chimerism and/or improved tolerance in kidney allograft recipients using nonmyeloablative conditioning and donor haematopoietic stem cell infusion (Sykes, 2009).

Another factor limiting successful islet engraftment is the inflammatory reaction that takes place in the liver after portal vein infusion of islets. This observation led to experiments of co-transplantation of islets with bone marrow-derived mesenchymal stem cells to take advantage of the anti-inflammatory action of these cells (Ito et al., 2010).

To improve islet vascularization is also a must for a better engraftment of islets. It has been shown that bone marrow-derived endothelial progenitor cells (EPC) isolated from peripheral blood specifically localize within sites of endothelial injury inducing a regenerative program. EPC are able to chimerize with donor vessels in transplanted organs, suggesting a putative role of these cells in graft revascularization (Schuh et al., 2008¸ Koopmans et al., 2006). EPC are recruited to the pancreas in response to islet injury and EPC-mediated pancreas neovascularization may facilitate the recovery of injured β cells improving islet allograft function (Mathews et al., 2004). In a murine model of islet transplantation, the increase of EPC in the peripheral circulation obtained by mobilization with granulocyte-macrophage colony-stimulating factor has been associated with higher vascular density and engraftment (Contreras et al., 2003). Therefore, the identification of factors able to enhance neoangiogenesis may increase the success of islet transplantation.

Another goal of combination therapies is to preserve islet function after detection of graft dysfunction (Froud et al., 2008). For this purpose it has been suggested, for instance, the use of exenatide, glucagon-like peptide synthetic analog, anti-tumor necrosis factor α agents or immunomodulatory therapy (Faradji et al., 2008; Froud et al., 2008). However, the recently released results of two trials addressing strategies of combination therapies are disappointing. The phase three trial based on combination of anti-CD25 mAb (Daclizumab) that blocks IL-2 signalling pathway in activated T cells without interferring with Treg, in combination with mycophenolate mofetil that bloks the *de novo* purine synthesis in T and B lymphocytes, reported no improvement of β cell preservation (Gottlieb et al., 2010). The anti-CD25 mAb was tested in another trial in association with exenatide. Also in this trial the improvement of β cell function was not observed (Rother et al., 2009). Several other phase II-III National Institutes of Health (NIH)–sponsored randomized trials in islet transplantation alone and in islet-after-kidney transplantation are currently under evaluation by the Clinical Islet Transplantation Consortium (http://www.citisletstudy.org/). An alternative to allow long term survival of islets after transplantation is the development of efficient encapsulation techniques aimed to guarantee immune-isolation, an adequate exchange of nutrients to islet cells and release of insulin (Calafiore et al., 2006). This kind of approach may protect from cell-mediated rejection of

implanted islets, although soluble mediators may still reach β cells and induce cell death. On the other hand, this strategy might allow full maturation of embryonic stem cells into glucose sensitive insulin secreting β cells. Indeed, human embryonic stem cells differentiated into pancreatic endoderm could be encapsulated in a protective device before transplantation (D'Amour et al., 2005; Kroon et al., 2008). Experiments in NOD mice demonstrated that encapsulated human β cell precursors may generate functional insulin producing cells after implantation improving diabetes (S.H. Lee et al., 2009). As an alternative, ongoing studies are evaluating the use of encapsulated porcine islets to correct type 1 diabetes (Elliott et al., 2007). Preliminary studies in humans showed long term survival of xenotransplantation of porcine neonatal islets and Sertoli cells using a technology to provide an immune protective environment (Valdes-Gonzales et al., 2005). A vascularized chamber model allowing tissue growth in threedimension under influence of hypoxia that triggers angiogenesis has been developped in mouse (Cronin et al., 2004) and rat (Mian et al., 2000; Tanaka et al., 2000). Vascularized chambers containing syngenic islets were used to improve glucose control in diabetic mice (Hussey et al., 2009). In this study islets were transplanted into prevascularized chambers implanted on the epigastric pedicle in the groin of diabetic mice resulting in a significant reduction in blood glucose levels and improvement of glycemic control. This study suggests that islet survival and function are enhanced by prevascularization of tissue engineering chambers before islet transplantation (Hussey et al., 2009). Opara et al. reported a new design of a bioartificial pancreas comprising islets co-encapsulated with angiogenic protein in permselective multilayer alginate-poly-L-ornithine-alginate microcapsules and transplanted in an omentum pouch in diabetic experimental animals (Opara et al., 2010). A great effort is invested to improve the encapsulation techniques to avoid clogging of the device that may reduce the influx of nutrients and glucose and impair the insulin efflux. Teramura & Iwata recently reviewed the obstacles and the new techniques to overcome these problems, such as conformal coating and islet enclosure with cells (Teramura & Iwata, 2010).

In consideration of the shortage of pancreata for islet transplantation and of the side effects of current immunosuppressive protocols, the research has been focused on the possible development of alternative sources of functional β cells. The ideal approach to overcome the current inadequate supply of human pancreatic islet cells for transplantation is the availability of an unlimited source of transplantable insulin-producing cells. The potential of adult and embryonic stem cells to generate islet cells as well as development of appropriate conditions for expansion and differentiation to β cells of pancreatic islet cell precursors or of cells that share common embryonic origin such as liver cells is under intense investigation (Mineo et al., 2009). Finally, xenogeneic islet transplantation remains a viable therapeutic option for the future (Ricordi, 2003).

5. In search for alternative sources of β cells

The consideration that stem cells play a crucial role to self-renewal in physiologic and pathologic conditions in the endocrine pancreas (Bouvens, 2006; Sarvetnick et al., 2007a, 2007b) prompted researchers to develop SC-based therapy to stimulate β cell regeneration.

In the last years, a variety of approaches have been employed to induce β cell neogenesis using pluripotent stem cells (embryonic stem cells or induced pluripotent stem cells), multipotent adult stem cells such as the hepatic oval cells or terminally differentiated cells such as the exocrine pancreatic cells (Yechoor & Chan, 2010). Brolén et al investigated the potential of

human embryonic stem cells to differentiate into β cells (Brolen et al., 2005). They found that signals from the embryonic mouse pancreas induce differentiation of human embryonic stem cells into insulin-producing β cell-like cells. Human embryonic stem cells (hESC) under two-dimensional growth conditions spontaneously differentiated in Pdx1(+)/Foxa2(+) pancreatic progenitors and Pdx1(+)/Isl1(+) endocrine progenitors but not in insulin-producing cells. The differentiation of β cell-like cell clusters required co-transplantation with the dorsal pancreas from mouse embryos. hESC-derived insulin(+) cell clusters exhibited several features of normal β cells, such as synthesis (proinsulin) and processing (C-peptide) of insulin and nuclear localization of key β cell transcription factors, including Foxa2, Pdx1, and Isl1 (Brolén et al., 2005). Insulin-producing islet-like cells were also generated from human embryonic stem cells under feeder-free conditions (Jiang et al., 2007). Cell aggregates formed in the presence of epidermal growth factor, basic fibroblast growth factor, and noggin were finally matured in the presence of insulin-like growth factor II and nicotinamide. The temporal kinetic of pancreas-specific gene expression was considerably similar to that of *in vivo* pancreas development. The final population contained cells representative of the ductal, exocrine, and endocrine pancreas. Ricordi et al reported a diabetes reversal in mice by embryonic-derived stem cells (Ricordi & Edlund, 2008). They showed that endodermal-derived embryonic stem cells were able to differentiate into cells that expressed typical pancreatic endodermal markers and, once transplanted in diabetic mice, these cells showed a diffuse staining for insulin, other hormones and markers of fully differentiated β cells (Ricordi & Edlund, 2008). The therapy with embryonic stem cells represents an exciting approach towards β cell regeneration and function. So far, the most promising data are from a group of NovoCell who demonstrated the generation of glucose-responsive insulin-secreting cells *in vivo* by pancreatic endoderm derived from human embryonic stem cells into immune-deficient mice (Kroon et al., 2008). However, several problems remain to be solved, such as the reproducibility of the advance, the protection of the engrafted cells against tumor formation and immune-protection that could be achieved by encapsulation devices. Recently, Matveyenko et al tried to reproduce and extend the studies of NovoCell by the implantation of human embryonic stem cell-derived pancreatic endoderm into athimic nude rats, analysing the metabolic parameters of insulin sensitivity and glucose-stimulated insulin secretion to verify the development of viable glucose-responsive insulin secreting cells. The implantation was assessed into the epididymal fat pads of the athymic nude rats or subcutaneously into Theracyte encapsulation devices for 20 weeks (Matveyenko et al., 2010). The data resulting from this study not completely confirmed the development of islet-like structures from human embryonic stem cells differentiated to pancreatic endoderm, since the extent of endocrine cell formation and secretory function was insufficient to be clinically relevant given that human C-peptide and insulin were detectable at very low levels, no increase in human C-peptide/insulin levels after glucose challenge and no development of viable pancreatic tissue or efficient insulin secretion by implantation in the encapsulation devices were present (Matveyenko et al., 2010).

Nevertheless, because the use of embryonic stem cells is burdened with the limited access to embryonic tissues and with the complex ethical concerns involved with the use of embryonic cells, more attention has been focused on adult stem cells. We found that stem cells derived from normal adult human liver (HLSC) with multiple differentiating capabilities distinct from those of oval stem cells may generate islet-like structures. HLSC expressed several MSC markers such as CD73, CD90, CD29, CD44 and the stem cell marker nestin (Herrera et al., 2006). At variance of MSC, HLSC did not express α-SMA and expressed liver tissue-specific proteins such as AFP, a marker of hepatocyte precursors, and

human albumin, suggesting a partial hepatocyte commitment. At variance with oval cells, the HLSC isolated from normal adult liver, did not express the CD34, c-kit and CK19 markers, and were pluripotent. Moreover, the HLSC were able to differentiate in appropriate conditions into pancreatic insulin producing cells (Herrera et al., 2006). When cultured in DMEM with high glucose content (23mM) for one month followed by 5-7 days of culture in the presence of 10 mM nicotinamide, HLSC formed small spheroid cell clusters positive for human insulin and Glut2 which is a glucose transporter that has been suggested to function as a glucose sensor in pancreatic β cells (L. Yang et al., 2002). Moreover, HLSC under this differentiating condition, were positively stained with the Zn-chelating agent dithizone, that is specific for the insulin containing granules (Shiroi et al., 2002). It has been recently shown that adult human pancreas contains rare multipotent stem cells that express insulin (Smukler et al., 2011).

Several studies demonstrate that the β cell phenotype can be induced both *in vitro* and *in vivo* by transfection of pancreatic transcription factors into the liver that develops from the same embryological origin of pancreas (Kojima et al., 2003; Lemaigre & Zaret, 2004; Nagaya et al., 2009).

The potential of bone marrow derived MSC has also been explored. Genetically modified MSC by recombinant PDX-1 adenovirus or by non-virus gene transfection, were able to express insulin sufficient to reduce blood glucose in the streptozotocin mouse model of diabetes (Y. Li et al., 2007). The differentiated PDX-1+ human MSC expressed multiple islet-cell genes such as neurogenin3 (Ngn3), insulin, GK, Glut2, and glucagon, produced and released insulin/C-peptide. After transplatation into STZ-induced diabetic mice the differentiated PDX-1+ human MSC induced within 2 weeks euglycemia and maintained it for at least 42 days. These findings suggest that appropriately modified MSC may allow enrichment of human β cells and represent a possible source for cell replacement therapy in diabetes (Y. Li et al., 2007).

Human umbilical cord blood contains several types of stem/precursor cells. Recent studies indicate the possibility to obtain multipotent stem cells from umbilical cord blood able to confer protection to β cells and to stimulate β cell neogenesis (Zhao & Mazzone, 2010). Islet-like cell clusters were obtained from MSC derived from humbilical cord vein. However, they secreted very low amounts of insulin in vitro (Chao, 2008). Denner et al showed that directed engineering of human cord blood stem cells may produce C-peptide and insulin (Denner et al., 2007).

A better understanding of the mechanisms of β cell regeneration may provide further insight for development of cell based therapeutic approaches. Besides the potential contribution of circulating and resident stem cells, the transdifferentiation of existing pancreatic cells such as pancreatic ductal/acinar cells into insulin secreting cells could be potentially exploited (Baeyens et al., 2005; Cantaluppi et al., 2008; Minami et al., 2005; Rosenberg, 1995).

The successful differentiation of stem cells into β cells may prove not to be easy. The secretion of insulin in physiological concentration in response to glucose concentration is the goal to be achieved. One of the more difficult tasks is to maintain an intact coupling of stimulus-secretion in order to obtain a regulated insulin secretion to keep physiological glucose concentration.

Another pre-requisite for β cell replacement therapy is to obtain a non limiting source of cells possibly derived from the patient himself to avoid the allo-immune response (Wagner et al., 2010). The possibility of reprogramming patient's own cells could be an

approach to these requirements. This was rendered possible by the discovery that induced pluripotent stem cells (iPS) can be generated from adult human somatic cells such as fibroblasts by transfection with selected genes encoding for transcription factors able to confer the characteristics of pluripotency to cells (Takahashi & Yamanaka, 2006; Yamanaka et al., 2007). iPS share the same differentiation potential of embryonic stem cells and they can differentiate into cells of different lineages including insulin producing cells. Tateishi et al tried to generate human iPS cells by retroviral expression of human Oct4, Sox2, Klf4 and c-Myc in the human foreskin fibroblast cells and tested the differentiation potential of human iPS cells into insulin secreting islet-like clusters, demonstrating that iPS cells derived from human skin cells can be differentiated into pancreatic islet-like cluster cells. These cluster cells were shown to contain C-peptide-positive and glucagon-positive cells and, more importantly, to release the C-peptide upon glucose stimulation (Tateishi et al., 2008). iPS cells were also generated from patients with type 1 diabetes by reprogramming their adult fibroblasts with three transcription factors (*OCT4, SOX2, KLF4*). The iPS generated from patients were shown to differentiate into insulin-producing cells (Maehr et al., 2009). A proof of principle for potential clinical applications of reprogrammed somatic cells in the treatment of diabetes type 1 or 2 was provided by Alipio et al who were able to reverse hyperglycemia in diabetic mouse models using iPS-derived pancreatic β like cells (Alipio et al., 2010). iPS could overcome the ethical issues and the immunogenicity of embryonic stem cells. The possibility to use iPS individually tailored for each patient is certainly appealing. However, several problems of bio-safety must be solved before iPS enter in clinical use. These include an aberrant or incomplete differentiation and a tumorigenic potential. Reprogramming cells typically requires integration of genes such as *c-MYC, OCT4 AND KLF4* that are known to be oncogenic and may favour development of tumors (Miura et al., 2009). Although after reprogramming the transgenes undergo silencing, it is always possible a reactivation. Therefore, the research in this field is actively searching for alternative strategies to induce pluripotency.

6. Stem cell therapy to counteract the autoimmune destruction of β cells

Assuming that we could generate pluripotent stem cell lines for each individual patient and that after infusion they would be able to restore the β cell mass, we still have to face the problem of recurrent autoimmunity. The autoimmune destruction of islet β cells by reactive T lymphocytes not only plays a role in the establishment of type 1 diabetes but also impairs an efficient regenerative process. Newly forming β cells show increased vulnerability to apoptosis induced by inflammatory cytokines (Meier et al., 2006), explaining the failure of β cell regeneration in type 1 diabetes. Therefore, newly generated β cells are systematically killed as they acquire the mature phenotype, thus impairing the regenerative attempts in type 1 diabetic patients. Furthermore, therapeutic intervention studies in new onset type 1 diabetes involving broadly immunosuppressive agents, such as cyclosporin A, failed to produce lasting remission, demonstrating the inherent tendency of the autoimmune effector response in humans to recur. Therefore, it is critical that any immunemodulatory therapy induces tolerance to β cell antigens, while minimizing detrimental effects on host defence. This is strengthened by recurrence of type 1 diabetes in syngenic pancreas transplantation (Sutherland et al., 1984).

Cell-based therapeutic strategies for immunemodulation that answer to the requirement of safety and effectiveness are under development. The intravenous use of humanized antibodies against CD3 (part of the T-cell receptor complex) soon after the initial onset of diabetic clinical symptoms decreased the insulin requirement compared to controls without this monoclonal antibody therapy. However, this therapy is burdened by several adverse effects (Keymeulen et al., 2005; Herold et al., 2002; McDevitt & Unanue, 2008).

Other approaches of immunemodulation included subcutaneous administration of heat-shock protein (Raz et al., 2001) or intravenous injection of rabbit polyclonal anti T-cell globulin (Saudek et al., 2004). In many of these studies a short term effective preservation of β cell function was observed; however, only few patients no longer required insulin treatment. Using human recombinant glutamic acid decarboxylase (GAD65) as therapeutic vaccine it was observed a preservation of C-peptide levels and a decrease in insulin dose requirement only in patients with very recent-onset of diabetes (Ludvigsson et al., 2008; Uibo and Lernmark 2008). Compelling evidence indicates the relevant role of Treg in the inhibition of autoimmune response. A decreased number and function of Treg have been reported in patients with type 1 diabetes (Roncarolo & Battaglia, 2007). Therefore, intervention to restore Treg function in diabetic patients is an attractive therapeutic strategy. Nevertheless, only few studies have shown re-establishment of Treg function in diabetes. Zhao et al demonstrated the possibility to correct functional defects of CD4+ CD62L Treg using human cord blood stem cells. This strategy allowed reversal of autoimmune diabetes in NOD mice (Y. Zhao et al., 2009) providing proof of principle that cord blood stem cells can correct function of Treg.

Treg may act by producing IL-10 and in particular transforming growth factor-β1 (TGFβ1), cytokines that are known to contribute to the induction of immunetolerance. It has been suggested that protection of newly generated β cells achieved by treatment with Treg depends on the formation of TGFβ1 cell ring surrounding pancreatic islands and conferring protection against autoimmune T lymphocytes inducing their apoptosis (Y. Zhao et al., 2009). In a pilot study in Type 1 diabetic children based on administration of autologous umbilical cord blood shortly after disease onset a lower level of HbA1c and a reduced insulin requirement was reported. However, none of the patients achieved insulin-treatment independency (Haller et al., 2007).

Recent studies raised great interest on the immunemodulatory potential of mesenchymal stem cells (Le Blanc & Pittenger, 2005). MSC can modulate several T cell functions exerting an immunesuppressive effect. MSC that lack MHC class II molecules and do not express key costimulatory molecules B7-1, B7-2, CD40 and CD40L, were shown to reduce the expression of several lymphocyte activation markers (Aggarwal & Pittenger, 2005). The mechanism has been related to the induction of an anti-inflammatory phenotype in dendritic cells, naive and activated T cells and NK cells, and to an increase of the regulatory T cell population. This results not only in the inhibition of T cell proliferation to polyclonal stimuli, but also in inhibition of naive and memory antigen-specific T cells response to their cognate peptide (Krampera et al., 2003). The MSC-induced suppression has been ascribed to several soluble factors, including hepatocyte growth factor (HGF), TGF-β1 and prostaglandin E_2 (PGE_2) (Aggarwal & Pittenger, 2005). Moreover, MSC have been shown to induce mature dendritic cells type 2 (DC2) to increase IL-10 secretion thus promoting anti-infammatory DC2 signaling. Several studies suggest that MSC improve the outcome of allogenic transplantation and hamper graft-versus-host disease (Le Blanc et al., 2004; Ringden et al.,

2006). Studies on the immunemodulatory potential of MSC in human type 1 diabetes are still lacking but it has been reported their regenerative potential in diabetic NOD/SCID mice leading to an increased number of pancreatic islets and β cells (R.H. Lee et al., 2006). We recently demonstrated that human allogenic bone marrow derived MSC can abrogate *in vitro* a pro-inflammatory Th1 response to islet antigen GAD in new onset type 1 diabetes, by impairing the production of IFN-γ and by inducing anti-inflammatory IL-4 production (Zanone et al., 2010). These data stimulate further studies on MSC immunemodulation in diabetes and open a perspective for immune-intervention strategies. The mechanisms of MSC interaction with the immune system cells are still controversial (van Laar & Tyndall, 2006; Abdi et al., 2008), and include reduction of the expression of lymphocyte activation markers, change of the cytokine profile of dendritic cells, naive and activated T cells and NK cells to an anti-inflammatory phenotype, and increase of the regulatory T cell population (Aggarwal & Pittenger, 2005; Le Blanc et al., 2004). In the reported study in type 1 diabetes, MSC induced in peripheral blood mononuclear cells (PBMC) of responder patients IL-4 producing cells and IL-4 secretion, suggesting a possible switch to an anti-inflammatory Th2 signalling of T cells (Zanone et al., 2010). Increased IL-4 secretion has been shown in studies of MSC cocultured with subpopulations of PHA-stimulated immune cells (Aggarwal & Pittenger, 2005) but not in studies of T cells activated by encephalitogenic peptide (Zappia et al., 2005). Lymphocyte activation is extremely complex and it is likely that several mechanisms are involved in the MSC-mediated immunesuppression and that the specific factors may depend on the lymphocyte population tested, the stimulus used, the timing of analysis and the context of the immune disease.

Further, inhibition of PGE_2 production abrogated the MSC-mediated IFN-γ suppression, indicating that PGE_2 secretion plays a key role in MSC-mediated immune effects, and the contact between MSC and PBMC enhances the production of prostaglandin E2 (Zanone et al., 2010). This observation suggests the requirement of both soluble factors and cell-contact in line with the interpretation that the immunemodulatory effects of MSC might require an initial cell-contact phase (Krampera et al., 2003). Nevertheless, the requirement of cell contact for MSC to operate their inhibition is a controversial issue, and the results reported in the literature may depend on the species and the type of stimulus.

Other studies in murine diabetic models on the regenerative capabilities of MSC, showed that injection of MSC into immunodeficient diabetic NOD/SCID mice resulted in the selective homing of MSC to pancreatic islets and in an increased number of pancreatic islets and functioning β cells (R.H. Lee et al., 2006). Further, MSC can be influenced to differentiate into cells with properties of the β cell phenotype, becoming more efficient after transplantation in mice (M. Zhao et al., 2008). Indeed, genetically modified MSC by recombinant Pdx-1 adenovirus or by non-virus gene transfection, were able to express insulin sufficient to reduce blood glucose in the streptozocin mouse model of diabetes (Karnieli et al., 2007; M. Zhao et al., 2008). More recently, allogenic MSC obtained from diabetes-prone as well as -resistant mice and injected into NOD mice, have been shown to delay the onset of diabetes or to reverse hyperglycemia (Fiorina et al., 2009). This study indicates that the beneficial effects observed could also be ascribed to the immunemodulatory capacities of MSC, as for other studies focusing on MSC-induced repair of cell injury (Abdi et al., 2008; Morigi et al., 2004; Duffield et al., 2005).

Studies on *ex vivo* expanded MSC to improve the outcome of allogenic transplantation and of acute graft-versus-host disease paved the way for the clinical use of MSC also in

autoimmune diseases. In experimental autoimmune encephalomyelitis, animal model of multiple sclerosis mediated by autoreactive T cells, injected MSC home to lymphoid organs where they cluster around T cells, and ameliorate the disease onset (Gerdoni et al., 2007). In this setting, MSC induce peripheral tolerance, impairing both the cellular and humoral arm of the encephalitogenic immune response, without evidence of transdifferentiation into neural cells (Zappia et al., 2005; Gerdoni et al., 2007). Furthermore, in a murine model of rheumatoid arthritis MSC have been demonstrated to exert an immunemodulatory effect by educating antigen-specific regulatory T cells (Augello et al., 2007). MSCs are also able to inhibit autoreactive T and B cells in experimental models of systemic lupus erythematosus (Deng et al., 2005). Overall, studies on murine models of type 1 diabetes (Fiorina et al., 2009; Madec et al., 2009) and multiple sclerosis (Zappia et al., 2005) as well as non-autoimmune diseases (Herrera et al., 2007) indicate that a key feature of MSC is their ability to selectively migrate into sites of injury, where they are likely to interact with activated T cells. Diabetogenic T cells are generated in pancreatic lymph nodes where they are introduced to antigens by dendritic cells. The preferential homing of MSC to pancreatic lympho nodes (Fiorina et al., 2009; Madec et al., 2009), supports the hypothesis that these cells could directly suppress autoreactive T cells *in vivo* within the pancreatic environment. Further, the desired therapeutic effects could be achieved by modulation of chemokines/receptors to promote the homing of MSC to specific anatomical sites (Sackstein et al., 2008). The first report on transplantation of human allogenic MSC, into a patient with autoimmune systemic sclerosis, indicates its safety and, notably, striking efficacy by selective immunesuppression and regeneration of impaired endothelial progenitors (Christopeit et al., 2008). Thus, results on the use of MSC infusion for treatment of severe graft-versus-host disease or autoimmune diseases (Lazarus et al., 2005; Fouillard et al., 2003; Le Blanc et al., 2004; Ringden et al., 2006; Christopeit et al., 2008) suggest a potential use in patients at risk of type 1 diabetes or at disease onset, to preserve or reduce loss of ß cells (Abdi et al., 2008; Staeva-Vieira et al., 2007).

7. MSC treatment of complications of diabetes mellitus

The ability of MSC to differentiate into tissue of mesodermal origin makes them attractive also as therapeutic agents for a number of complications of diabetes, including cardiomyopathy, nephropathy, polyneuropathy and diabetic wounds. MSC have been shown to differentiate into several cell types, including cardiomyocytes, endothelial cells, neurons, hepatocytes, epithelial cells and adipocytes, characteristics coupled with capacity of self renewal.

Chronic hyperglycemia is responsible for myocardial remodelling leading to ventricular dysfunction with hypertrophy and apoptosis of cardiomyocytes, microcirculatory defects, altered extracellular matrix and matrix metalloproteinasis (Jesmin et al., 2003). MSC can induce myogenesis and angiogenesis by different mitogenic, angiogenic and antiapoptotic factors, such as VEGF, IGF-1 and HGF (Zhang et al., 2008). In a diabetic rat model, intravenous MSC have been shown to improve cardiac function, potentially by differentiation into cardiomyocytes and improvement of myogenesis and angiogenesis. Metalloproteinase activity was also modulated, leading to increased arteriolar density and decreased collagen volume. Cardioprotection is probably more mediated by release of paracrine factors by MSC. These include VEGF, HGF, Bcl-2, Hsp20, activation of Akt

(Wang et al., 2009), which can affect remodelling, repair and neovascularization. Intravenous autologous MSC in post-infarction patients have indeed been shown to reduce episodes of ventricular tachycardia and to increase ventricular ejection fraction (R.H. Lee et al., 2010).
Diabetic limb ischemia could also be improved by MSC-derived pro-angiogenic factors (Comerota et al., 2010).
MSC have also been used for treatment of diabetic nephropathy in NOD/SCID and streptozocin C57B1/6 mice (R.H. Lee et al., 2006). The injected cells engraft in damaged kidneys, potentially differentiate into renal cells and endothelial cells and can regulate the immune response. This resulted in improved kidney function and regeneration of glomerular structure. MSC, however, were unable to proliferate; therefore, it is conceivable that MSC contribute to the repair by releasing paracrine factors that promote neovasculatization and limit cytotoxic injury.
As for diabetic polyneuropathy, in diabetic rats, intramuscular injection of MSC led to increased ratio of capillaries to muscle fibers, improvement of hyperalgesia and function of neural fibers. MSC settled in the gap between muscle fibers at the transplanted site, and produced VEGF and basic bFGF, without differentiating into neural cells (Shibata et al., 2008).
By releasing paracrine factors and by differentiation into photoreceptor and glial-like cells in the retina, transplanted MSC have also been shown to improve the integrity of the blood-retinal barrier, ameliorating diabetic retinopathy in streptozocin diabetic rats (Z. Yang et al., 2010).
Prolonged and uncompleted wound healing can complicate the diabetic condition. Injection of MSC in animal models of diabetes improved wound healing, with increase of collagen levels and of wound-breaking strength, together with increased levels of TFGβ, KGF, EGF, PDGF, VEGF, all involved in repair (Wu et al., 2007). Besides these paracrine effects MSC were shown to differentiate and regenerate damaged epithelium.
Limitation of the potential therapeutic use of MSC also for diabetic chronic complications are, at present, mainly the poor engraftment and the limited differentiation under *in vivo* conditions, together with the potential differentiation into unwanted mesenchymal lineages.

8. Conclusion

A number of issues should be addressed before a cell based therapy may come to a clinical setting. The first challenge is to define which kind of cells are more suitable for β cell substitution. This implies to develop efficient strategies of stem cell differentiation that lead to cells that produce and secrete insulin in physiological amounts under the control of glycemia. Moreover, safety of a cell based therapy remains a critical point, as any precursor or stem cell types might induce tumor formation. Whether the achievement of fully differentiated cells would reduce this risk remains to be proved. Another relevant point is to define the strategies that allow an immune modulation to avoid the recurrence of autoimmune destruction of newly formed β cells.

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16

Prevention of Diabetes Complications

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1. Introduction

The constellation of abnormalities caused by insulin deficiency is called diabetes mellitus. The cause of clinical diabetes is always a deficiency of the effect of insulin at the tissue level. Type I diabetes or insulin- dependent diabetes mellitus (IDDM), is due to insulin deficiency caused by autoimmune destruction of the B cell in the pancreatic islets, and it accounts for 3-5 % of cases and usually presents in children. Type 2 diabetes, or non-insulin-dependent diabetes mellitus (NIDDM), is characterized by the dysregulation of insulin release from the B cells, along with insulin resistance in peripheral tissues such as skeletal muscle, brain, and liver. Type 2 diabetes usually presents in overweight or obese adults.
The incidence of diabetes in the human population has reached epidemic proportions worldwide and it is increasing at the rapid rate.150 million people in 2000, which is predicted to rise to 220 million in 2010.
In animals, it can be produced by pancreatectomy; by administration of alloxan, streptozocin, or other toxins that in appropriate doses cause selective destruction of the beta cells of the pancreatic islets; by administration of drugs that inhibit insulin secretion; and by administration of anti-insulin anti-bodies. Strains of mice, rats, hamsters, guinea pigs, miniature swine, and monkeys that have a high incidence of spontaneous diabetes mellitus have also been described.
Diabetes is characterized by polyuria, polydipisa, weight loss in spite of polyphagia, hyperglycemia, glycosuria, ketosis, acidosis, and coma. Widespread biochemical abnormalities are present, but the fundamental defects to which most of the abnormalities can be traced are 1) reduced entry of glucose into various peripheral tissues and 2) increased liberation of glucose into the circulation from the liver. Therefore there is an extracellular glucose excess and, in many cells, an intracellular glucose deficiency a situation that has been called starvation in the midst of plenty. Also, the entry of amino acids into muscle is decreased and lipolysis is increased.

2. Diabetes complication

Diabetic complications are divided to two parts: 1) metabolic complication and 2) vascular complication.

2.1 Metabolic complication

Obesity is increasing in incidence, and relates to the regulation of food intake and energy balance and overall nutrition. It is special relation to disordered carbohydrate metabolism

and diabetes. As body weight increase, insulin resistance increase, that is, there is a decreased ability of insulin to move glucose into fat and muscle and shut off glucose release from liver. The liver takes up glucose from the bloodstream and stores it as glycogen, but because the liver contains glucose 6- phosphates it also discharges glucose into the blood–stream. Insulin facilitates glycogen synthesis and inhibits hepatic glucose output. When the plasma glucose is high, insulin secretion is normally increased and hepatic glucogenesis is decreased. This response dose not occurs in type I and II diabetes.

When plasma glucose is episodically elevated over time, small amounts of hemoglobin A are nonenzymatically glycated to from HbA1c. Careful control of the quently HbA1c level is measured clinically as an integrated index of diabetic control for the 4 to 6 weeks period before the measurement. Many studies showed that the mean HbA1c value was a good predictor of ischemic heart disease. In particular, the multivariate analysis showed that per each 1% increment in HbA1c there was a 10% increase in the risk of coronary heart disease. Some studies believed that 1% reduction in HbA1c level led to a 16% reduction in the occurrence of myocardial infarction.

Peripheral neuropathy, often expressed as hypersensitivity to painful stimuli, is among the most common complications of diabetes that develops in up to 60% of patients. It occurs in both type I and II diabetes and its incidence is linked to duration of disease. Neuropathic pain is a chronic or persistent pain characterized by alterations in pain perception, enhanced sensitivity to noxious stimuli (hyperalgesia) and abnormal pain sensitivity to previously non-painful stimuli (allodynia). Though the pathophysiology of neuropathy in diabetes has not been fully elucidated, hyperglycemia induced by diabetes is though to contribute to its development and maintaining good glycemic control could restrict the onset and progression of diabetic neuropathy.

2.2 Vascular complication

Hyperglycemia has a direct, harmful effect on the cardiovascular system requires, at the very least, a link between acute hyperglycemia and one or more risk factors for cardiovascular disease (CVD). Associated with obesity there is hyperinsulinemia, high circulating triglyceride and low HDL, and accelerated development of atherosclerosis. In diabetes, the plasma cholesterol level is usually elevated and this plays a role in the accelerated development of the atherosclerotic vascular disease that is a major long-term complication of diabetes in humans.

As usual diabetes is characterized by a high incidence of CVD, and poor control of hyperglycemia appears to play a significant role in the development of CVD in diabetes. Recently, there has been increasing evidence that the postprandial state is an important contributing factor to the development of atherosclerosis. In diabetes the postprandial phase is characterized by a rapid and large increase in blood glucose levels, and the possibility that these postprandial hyperglycemic spikes may be relevant to the pathophysiology of the late diabetes complications.

Insulin resistance (IR) has profound, negative effects on the function of arteries and arterioles throughout the body. In addition to the obvious link between IR and the development of type 2 diabetes, IR-associated dysfunction of resistance vessels is associated with arterial hypertension and vascular occlusive diseases. IR affects arteries and arterioles at both the endothelium and smooth muscle levels. For example, IR causes reduced responsiveness of vascular smooth muscle to dilator agents; predominantly due to impaired potassium channel function.

Vascular disease is one of the complicating features of diabetes mellitus. Several prospective studies have indicated that hypertension in diabetic patient's takes place at a rate more than twice compared to the normal population. The hypertension is also considered an independent risk factor for cardiovascular mortality in patients with diabetes. It has been suggested that alterations in the reactivity of blood vessels to neurotransmitters and circulating hormones are responsible for the cardiovascular complications of diabetes. Some studies showed that Ca/Mg ratio is a marker of vascular tone; its increase represents increased vascular reactivity and atherogenic risk. Atherogenic lesion is poorly correlated with serum cholesterol level and is highly dependent on plasma magnesium level and Ca/Mg ratio. Pervious studies showed that Ca/Mg ratio increase in diabetic case. Endothelial function is altered early in diabetes. It has been demonstrated that in diabetic subjects, the vasodilating response to stimuli is diminished and that this anomaly is related to glycemic control. In vivo studies have demonstrated that hyperglycemic spikes induce, in both diabetic and normal subjects, an endothelial dysfunction. This effect of hyperglycemia is probably linked with a reduced production/bioavailability of nitric oxide (NO), since hyperglycemia-induced endothelial dysfunction is counterbalanced by arginine. Furthermore, it is very interesting that a rapid decrease of flow-mediated vasodilation has been shown in the postprandial phase in type II diabetes patients and that the decrease correlated inversely with the magnitude of postprandial hyperglycemia.

3. Prevention of diabetes complications

Type I diabetes usually develops before the age 40, patients with this disease are not obese and they have a high incidence of ketosis and acidosis. Various anti-B cell antibodies are present in plasma, but the current thinking is that type I diabetes is primarily a T lymphocyte-mediated disease. But type II diabetes is the most common type of diabetes and is usually associated with obesity. It usually develops after age 40 and is not associated with total loss of the ability to secrete insulin. It has an insidious onset, is rarely associated with ketosis, and is usually associated with normal beta-cells morphology and insulin content if the beta-cells have not become exhausted. So it seems we should look for different methods for prevention of type I and II diabetes.

3.1 Diabetes diet

Several lifestyle factors affect the incidence of type 2 diabetes. Obesity and weight gain dramatically increase the risk, and physical inactivity further elevates the risk, independently of obesity. Cigarette smoking is associated with a small increase and moderate alcohol consumption with a decrease in the risk of diabetes. In addition, a low-fiber diet with a high glycemic index has been associated with an increased risk of diabetes, and specific dietary fatty acids may differentially affect insulin resistance and the risk of diabetes.

Excess body fat is the single most important determinant of type II diabetes. Weight control would be the most effective way to reduce the risk of type II diabetes, but current strategies have not been very successful on a population basis, and the prevalence of obesity continues to increase. The public generally does not recognize the connection between overweight or obesity and diabetes. Thus, greater efforts at education are needed.

Low-fat vegetarian and vegan diets are associated with reduced body weight, increased insulin sensitivity, and reductions in cardiovascular risk factors. The potential cardiovascular benefits of vegetarian and vegan diets may be especially important for

individuals with diabetes, for whom cardiovascular disease is a main cause of premature mortality; the effects of such diets on cardiovascular risk factors appear to be similar in individuals with and without diabetes.
Prior studies have shown that near-vegetarian diets reduce the need for insulin and oral medications in individuals with type 2 diabetes. We previously reported that in individuals with type 2 diabetes, a low-fat, vegan diet was associated with improved glycemic control, weight loss, and improved plasma lipid control during a 22-wk study period. What is particularly critical in diabetes management is long-term improvement in clinical measures, particularly glycemia and cardiovascular risk factors. Well-planned low-fat vegan diets are nutritionally adequate and, in research studies, have shown acceptability comparable with that of other therapeutic diets, suggesting they are suitable for long-term use.

3.2 Immunosupression drug

If type one diabetic patient give immunosupression drugs like cyclosporine ameliorate early in the disease, before all beta cells are lost can be useful for prevention of disease. But chose the low fat and low carbohydrate diets could be useful for prevention of type 2 diabetes.

4. Magnesium

Some studies indicated that magnesium is a novel factor implicated in the pathogenesis of the complication of diabetes. Magnesium plays a fundamental role as a cofactor in various enzymatic reactions of energy metabolism. Magnesium is a cofactor in cell membrane glucose-transporting mechanisms, as well as in various enzymes in carbohydrate oxidation. It is also involved, at multiple levels, in insulin secretion, binding and activity. Magnesium deficit has been described in patients with type I diabetes. Hypomagnesemia can also be the cause or a result of diabetes complications. If it is followed by diabetes, osmotic diuresis may play a role in the mechanisms responsible for magnesium deficiency. Magnesium loss may be linked to the development of diabetes complications via a reduction in the rate of inositol transport and its subsequent intracellular depletion that might enhance the development of complications. Studies showed that the administration of magnesium corrected hyperglycemia and has brought blood glucose back to normal levels within 24 h of its administration. Moreover, magnesium appears to have some reparative effect on the pancrease of diabetic case. Accordingly, during long-term treatment, pancreatic repair may have an effective role in the control of plasma glucose levels. Magnesium also is a necessary cofactor for many enzymes which is involved in lipid metabolism. Mg-deficiency enhances catecholamine secretion which result in an increase in lipolysis and blood plasma magnesium has been shown to decrease when lipolysis is increased. Enhancement in lipolysis and subsequent elevation of plasma free fatty acids levels may lead to an increase in hepatic VLDL and triglycerides synthesis and secretion and elevated plasma triglyceride concentration. The hepato-biliary pathway is the main rout for removal of cholesterol from the body. Bile flow is significantly lower in Mg-deficient subject than in controls and the cholesterol concentration in bile is decreased. Magnesium administration could decrease triglycerides, cholesterol and LDL cholesterol and also increased HDL cholesterol. The decrease in serum triglycerides was associated with the change in serum total Mg concentration. Other supporting evidence is accumulating for the role of magnesium in the modulation of serum lipids and lipids uptake in macrophages. Studies showed that increase

in plasma endothelin I due to magnesium deficiency and a direct effect of magnesium deficiency on vascular smooth muscle are involved in the elevation of vascular tone in diabetic pateint. Elevated vascular tone can contribute to increased blood pressure. Some studies have observed that systolic and diastolic blood pressure and mean arterial blood pressure in Mg-treated chronic diabetic subject are lower than in chronic diabetic. The administration of magnesium can decrease vascular bed sensitivity to phenylephrine and decrease Ca/Mg ratio. Studies also showed that magnesium decreases collagen thickness, intima/media thickness and the lumen/ media ratio in aorta. This suggests that the administration of magnesium can decrease blood pressure and prevent vascular morphological changes and decrease in vascular sensitivity to neurotransmitter. Hemoglobin deficiency is observed in diabetic subjects. This can probably be explained by the inhibition of ð-aminolevulinate dehydratase (ð-ALA-D) in diabetes. Studies have found that this enzyme is inhibited by glycation of the active site lysine residue involved in Schiff's base formation with the first ð-ALA-D molecule. Magnesium administration reduces this glycation via blood glucose reduction and, thus, prevents hemoglobin deficiency. So it seems that magnesium administration may play in the management of diabetes and the prevention of its vascular complications in diabetic patients.

5. Glucagone-like peptide-1 (GLP-1)

Type I diabetes is a complex disease that results from an autoimmune T-lymphocyte-dependent islet infiltration and destruction of islet beta- cells, with consequent insulin deficiency and dependence on exogenous insulin treatment. A strikingly decreased functional beta- cell mass owing to apoptosis constitutes the histopathological hallmark of the disease at diagnosis. Recently, strategies employing beta-cell growth factors to enhance functional beta-cell mass and restore insulin secretion have been proposed for the treatment and prevention of diabetes. One such promising beta-cell growth factor identified is glucagone-like peptide-1 (GLP-1). GLP-1 is an insulinotropic hormone that is secreted from intestinal L-cell in response to nutrient ingestion and promotes nutrient absorption via regulation of islet hormone secretion. GLP-1 receptor is expressed mainly by pancreatic beta-cells, and to some extent in other tissues like lung, kidney and brain. GLP-1 enhances pancreatic islet beta-cell proliferation and inhibits beta-cell apoptosis in a glucose-dependent fashion. Other actions of GLP-1 are to decrease glucagons secretion and gastric emptying. Together, all these actions tend to lower the plasma glucose concentration and to limit plasma glucose rises with meals. GLP-1 is another gastrointestinal hormone that is also expressed in the hypothalamus and brainstem. Their CNS actions are to decrease food intake, decrease water intake, and increase diuresis. However, native GLP-1 has a short circulating half-life (less than 2 min) that results mainly from rapid enzymatic inactivation by dipeptidyl-peptidase IV (DPP- IV), and/ or renal clearance. Therefore, continuous subcutaneous infusion by pump is necessary to maintain GLP-1 action. DPP- IV-resistant GLP-1 analogues and other formulations appear to be promising therapeutic drug candidates for the treatment and prevention of diabetes, but these peptides require once or twice-daily injections and/or combination therapies with oral diabetic medications. Scientifics recently developed a novel GLP-1 fusion peptide consisting of the active human GLP-1 molecule and the murine IgG1 constant heavy-chain (IgG-Fc). Plasmid-based, electroporation-enhanced intramuscular gene therapy with GLP-1/IgG-Fc improved insulin production and normalized glucose tolerance in type one or two diabetes.

6. Gama amino butyric acid (GABA)

Gama amino butyric acid (GABA) is an important neurotransmitter which was initially identified in the central nervous system and is also found in islet beta-cells. GABA has an important role in pathogenesis of diabetes. Excessive secretion of glucagon is a major contributor to the development of diabetic hyperglycemia. Secretion of glucagon is regulated by various nutrients, with glucose being a primary determinant of the rate of alpha-cell glucagon secretion. The intra-islet action of insulin is essential to exert of insulin, glucose is not able to suppress glucagons release in vivo. However, the precise mechanism by which insulin suppresses glucagon secretion from alpha-cells is unknown. Studies showed that insulin induces activation of GABAA Akt kinase-dependent pathway. This leads to membrane hyperpolarization in the alpha-cells and, ultimately, suppression of glucagon secretion. Researchers propose that defects in this pathway contribute to diabetic hyperglycemia. It is well known that the secretion of glucagon is abnormal in human type I diabetes patients. The patients do not secrete glucagon in response to hypoglycemia and they have an exaggerated response of glucagon to stimuli such as arginine infusion and a protein meal. In studies of patients with type I diabetes there are indications of an increase in alpha cell numbers. GABA decreases in diabetic patients. Some studies indicated that a reduction in cellular GABA level is more sensitive than insulin as a marker for the presence of dead beta-cells in isolated preparations. Pancreatic GABA content also rapidly decreased after diabetes induction and remained unaffected by 12 h of hyperglycemia. It seems that GABA therapy can has some beneficial effect to prevention or treatment type I diabetes.

7. Antioxidants

Increasing evidence in both experimental and clinical studies suggests that oxidative stress play a major role in the pathogenesis of both types of diabetes mellitus. Diabetes is usually accompanied by increased production of free radicals or impaired antioxidant defenses. Mechanisms by which increased oxidative stress is involved in the diabetic complication are partly known, including activation of transcription factors, advanced glycated end products (AGEs), and protein kinase C.

Excessively high levels of tree radicals cause damage to cellular proteins, membrane lipids and nucleic acids, and eventually cell death. Various mechanisms have been suggested to contribute to the formation of these reactive oxygen-free radicals. Glucose oxidation is believed to be the main source of free radicals. In its enediol form, glucose is oxidized in a transition-metal-dependent reaction to an enddiol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals. The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded by catalase or glutathione peroxidase, and in the presence of transition metals, can lead to production of extremely reactive hydroxyl radicals. Superoxide anion radicals can also react with nitric oxide to form reactive peroxynitrite radicals. Hyperglycemia is also found to promote lipid peroxidation of LDL by a superoxide- dependent pathway resulting in the generation of free radicals. Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to the formation of Amadori product and then advanced glycation endproducts (AGEs). These AGEs, via their receptors (RAGEs), inactivate enzymes and alter their structures and functions, promote free radical formation, and quench and block

antiproliferative effects of nitric oxide. By increasing intracellular oxidative stress, AGEs activate the transcription factor NF-kB, thus promoting up-regulation of various NF-kB controlled target genes. NF-kB enhances production of nitric oxide, which is believed to be a mediator of islet beta cell damage.

Considerable evidence also implicates activation of the sorbitol pathway by glucose as a component in the pathogenesis of diabetic complications, for example, in lens cataract formation or peripheral neuropathy. Efforts to understand cataract formation have provoked various hypotheses. In the aldose reductase osmotic hypothesis, accumulation of polyols initiates lenticular osmotic changes. In addition, oxidative stress is linked to decreased glutathione levels and depletion of NADPH levels. Alternatively, increased sorbitol dehydrogenase activity is associated with altered NAD^+ levels, which results in protein modification by nonenzymatic glycosylation of lens proteins.

Mechanisms linking the changes in diabetic neuropathy and induced sorbitol pathway are not well delineated. One possible mechanism, metabolic imbalances in the neural tissues, has been implicated in impaired neurotrophism, neurotrsnsmission changes, Schwann cell injury, and axonopathy.

While on the one hand hyperglycemia engenders free radicals, on the other hand it also impairs the endogenous antioxidant defense system in many ways during diabetes. Antioxidant defense mechanism involves both enzymatic and nonenzymatic strategies. Common antioxidants include the vitamins A, C and E, antioxidant minerals (copper, zinc, manganese, and selenium), and the cofactors (folic acid, vitamins B1, B2, B6, B12). They work in synergy with each other and against different types of free radicals. Vitamin E suppresses the propagation of lipid peroxidation; vitamin C with vitamin E inhibits hydroperoxide formation; metal complexing agents, such as penicillamine, bind transition metal involved in some reactions in lipid peroxidation and inhibit Fenton and Haber-weiss-type reactions; vitamins A and E scavenge free radicals.

8. Herbal medicine

Recently, the search for appropriate hypoglycemic agents has been focused on plants. Many herbal medicines have been recommended for the treatment of diabetes. Plant drugs are frequently considered to be less toxic and free from side effect than synthetic ones. The leaf of Psidium guava, Teucrium polium, Cinnamon and Garlic are used traditionally in many countries to manage, control and treat of diabetes. Some recent studies have shown that administration of Psidium guava or Teucrium polium leaves decrease blood glucose via enhance insulin secretion.

Psidium guajva Linn., commonly known as guava, is a native plant in tropical American and has long been naturalized in south east Asia and in south of Iran. Different parts of the plant are used in traditional medicine for the treatment of various human aliments such as wound, ulcers, bronchitis, cyesores and diarrhea. In folklore guava has been used for a long time as a medicinal herb to cure diabetes mellitus. Many people in some countries including Japan, Taiwan and Iran boil guava leaves in water and drink the exact as a folk medicine for diabetes and hypertension. Psidium guajava leaves have a beneficial effects on diabetes metabolic syndrome and vascular complications.

Photochemical analysis of Psidium guajava leaves have revealed the presence of flavonoids, which include quercetin and its derivatives (guajaverin, isoquercitrin, hyperin, quercitrin, avicularin), morin and its derivatives (morin-3-O-α-L-lixopyranoside and morin-3-O-α-L-

arabopyranoside), rutin, myricetin, luteolin and kaempferol. The leaves of the plant have also been shown to contain essential oil, fixed oil, volatile oil, saponin, resin, tannin, triterpenoids, asiatic acid and ellagic acid.

The relaxant effect of Psidium guajva Linn., on endothelium-intact aortic rings were only partially inhibited by N-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, suggesting that the vasorelaxant effect of Psidium guajva Linn., on aortic rings is probably mediated via both endothelium-derived relaxing factor (EDRF)-dependent and EDRF-independent mechanisms but it seems this mechanism is not follow in diabetic rat vessel.

Teucrium polium L. is one of 300 species of the genus Teucrium and found mainly in the Mediterranean and Western Irano-Turanian sphere. It is widely distributed in Iran, Jordan and Palestine. The leaves 1-3 cm long, are sessile, oblong or linear, the stems are ending in a shortly paniculate or corymbose infoflorescences, corolla is white or pale cream colored. Several researchers have evaluated Teucrium polium grown in different geographic origin and it has flavonoids and iridoids. Hypoglycemic activity has been reported - in addition to the flavonoids- also for the volatile oils. Traditionally, especially in the Mediterranean countries and in Iran, Teucrium polium, is used for its antispasmodic and hypoglycemic activities by the native inhabitants and recommended by the herbalists. Anti-inflammatory, anti-hypertensive, antinociceptive, anti-ulcer and anorexic effects are other activities reported. Some investigators have reported reduction in blood glucose concentrations of animal diabetic model after treatment with a single i.v., i.p. and oral dose of Teucrium polium aqueous decoction. Some Iranian researchers have observed significant decrease in blood glucose in animal diabetic model after six weeks of consecutive oral treatment with ethanol/ water extract.

Spices such as Cinnamon display insulin-enhancing activity in vitro. Cinnamon can improve glucose metabolism and the overall condition of individuals with diabetes not only by hypoglycemic effects but also by improving lipid metabolism, antioxidant status, and capillary function. Aqueous extracts from Cinnamon have also been shown to increase in vitro glucose uptake and glycogen synthesis and to increase phosphorylation of the insulin receptor; in addition, these Cinnamon extracts are likely to aid in triggering the insulin cascade system. Because insulin also plays a key role in lipid metabolism, consumption of Cinnamon would lead to improved glucose and blood lipids in vitro. The mechanism of the effect of Cinnamon on glucose and blood lipids is not completely understood but the researchers believe that extracts of Cinnamon activated glycogen synthase, increased glucose uptake, and inhibited glycogen synthase kinase-3β. Extracts of Cinnamon also activated insulin receptor kinse and inhibited dephosphorylation of the insulin receptor, leading to maximal phosphorylation of the insulin receptor. All of these effects would lead to increased insulin sensitivity. The extract of Cinnamon also has function as potent antioxidants, which would lead to additional health benefits of this substance.

Garlic was known to be effective in decreasing, cholesterol and can inhibit LDL-Oxidation. Many clinical trials have been conducted to determine the lipid-lowering effects of fresh garlic and garlic supplements. Garlic consumption also can decrease blood glucose in diabetic patients and has beneficial effect on diabetic vessel. It seems that daily Garlic consumption can be useful to prevent of diabetes.

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17

The Enigma of β-Cell Regeneration in the Adult Pancreas: Self-Renewal Versus Neogenesis

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1. Introduction

The pancreas is constituted by two distinctly different tissues: the exocrine component, i.e., pancreatic acinar cells that secrete digestive enzymes; and the endocrine component, the islets of Langerhans, constituted by hormone secreting cells. In the islet, the α-cells produce glucagon; the β-cells, insulin; the δ-cells, somatostatin; γ-cells, pancreatic polypeptide (Figure 1). Diabetes is caused either by an absolute (type 1) or relative (type 2) defect of insulin-producing β-cells in the pancreas. Therefore, regardless of the different pathogenesis, diabetes is the perfect candidate for cell replacement therapy. Currently the two available alternatives for β-cell replacement therapy are whole pancreas or isolated islet transplantation (Shapiro et al., 2000). However, these approaches are severely limited by a shortage of human organ donors and the need of lifelong immunosuppressive therapy. In the absence of other clearly suitable and renewable sources of surrogate β-cells, an alternative strategy to exogenous cell replacement therapy might be fostering endogenous β-cell regeneration. Therefore, knowledge of the mechanisms regulating β-cell plasticity in both embryonic and adult life, as well as in pathological conditions, is of particular interest.
During pancreatic development, β-cells derive from a population of endocrine precursors arising from the pancreatic epithelium (Gittes, 2009). Activation of cell-specific transcription factors guides the initially multipotent progenitors and determines their differentiation into mature β-cells. The final size of the endocrine pancreas is limited by the size of the progenitor cell pool in the developing pancreatic bud (Stanger et al., 2007). After birth, most β-cells are considered quiescent, however, it has been shown that the β-cell mass can adaptively expand under some physiologic or pathologic circumstances, such as pregnancy and obesity, both in mammals and rodents (Bernard-Kargar & Ktorza, 2001).
In mouse models there is also evidence that the pancreas preserves the ability to regenerate its β-cell mass in response to several non-physiological injuries, such as selective chemical destruction or surgical excision (Trucco, 2005; Thorel, 2010). Furthermore, in non-obese diabetic mice (NOD) it has been shown that recovery of sufficient endogenous insulin

production is possible via combination of strategies involving reversal of the autoimmune attack (Zorina et al., 2003; Kodama et al., 2003; Suri et al., 2006; Chong et al., 2006; Nishio et al., 2006).

In humans it is still debated whether this recovery is possible and, if so, to what extent it is feasible. Spontaneous recovery of β-cell function has been reported in patients with recent onset type 1 diabetes, suggesting that β-cells can regenerate despite underlying autoimmunity (Karges et al., 2004, 2006; Meier et al., 2006a; Butler et al., 2007). Additionally, the observation that people with long-standing type 1 diabetes still possess β-cells despite their destruction by the enduring autoimmunity and glucotoxicity, suggests that new β-cell formation might occur throughout life [Meier et al., 2005]. However, it remains largely unclear through which molecular and cellular mechanisms it occurs.

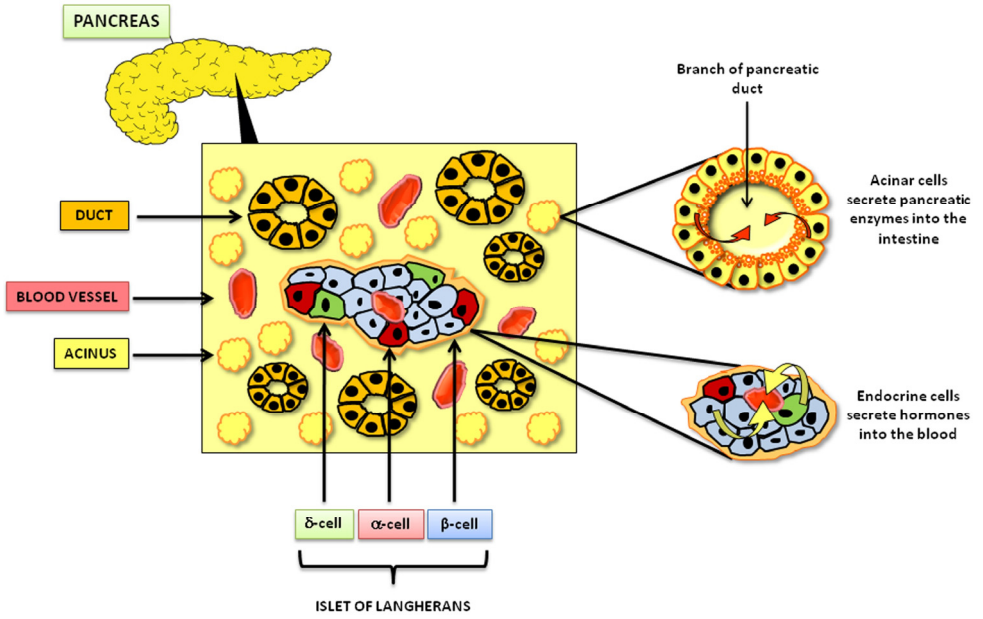


Fig. 1. Cells of the pancreas

The pancreas houses two different tissues. Its bulk is comprised of exocrine tissue, which is made up of acinar cells. These cells secrete pancreatic enzymes delivered to the intestine to facilitate the digestion of food. Scattered throughout the exocrine tissue are many thousands of clusters of endocrine cells known as islets of Langerhans. Within the islet, α-cells produce glucagon; β-cells, insulin; δ-cells, somatostatin; and γ-cells, pancreatic polypeptide — all of which are delivered into the blood stream.

2. Can the endocrine pancreas regenerate? Models of artificially induced diabetes

To address the question whether the pancreas possesses the ability to regenerate, several models have been used to artificially reduce the β-cell mass in order to stimulate a

pancreatic response. Most of these models allow exploring the plasticity of the pancreas in the absence of concurrent autoimmunity, which might prevent or block any attempt of β-cell restoration.

2.1 Partial pancreatectomy

Partial pancreatectomy (>90% removal) was shown to induce limited re-growth of the remnant organ in rats (Pearson et al., 1977). In comparison to liver regeneration second to partial hepatectomy, subtotal pancreatectomy is followed only by a limited regenerative growth that is proportional to the size of the excision. In addition, regeneration is mostly related to the exocrine tissue, while the endocrine part was shown to rescue, at its best, only approximately 30% of its initial mass (De Leon et al., 2003). The extent of the surgical intervention seems to be important, and could explain discrepancies in some reports describing absent (Dor et al., 2004) or vigorous pancreatic regeneration [Bonner-Weir et al., 1993] after 70% and 90% organ resection, respectively. In addition, hyperglycemia, which is present only in the latter case, could act as co-stimulator.

2.2 Pancreatic duct ligation

Pancreatic duct ligation (PDL) was also used to determine obstruction and consequently local inflammation and stimulation of pancreatic regeneration. During the first week post-ligation an increase in the β-cell number and the presence of intermediate ductal/endocrine (Wang et al., 1995) or acinar/endocrine phenotypes (Bertelli & Bendayan, 1997; Inada et al., 2008) has been observed. Additional stimulation of the expansion of the β-cell mass following PDL can be achieved by gastrin infusion (Rooman et al., 2002).

2.3 Wrapping of the pancreas with cellophane

Wrapping the pancreas with cellophane has also been used to induce islet neogenesis from ducts, and it has been reported to reverse streptozotocin-induced diabetes in hamsters (Rosenberg et al., 1996).

2.4 Selective β-cell destruction

Selective β-cell destruction can be obtained by chemical ablation with streptozotocin or alloxan, alone and in combination with pancreatectomy (Finegood et al., 1999; Wang et al., 1996; Rood et al., 2006). Streptozotocin (STZ) is a drug that leads to cell death by DNA alkylation, while alloxan is a generator of oxygen free radicals causing extensive DNA damage. Adult mice rendered diabetic with a high dose of STZ or alloxan are unable to recover endogenous β-cell function (Szkudelski, 2001). Interestingly, β-cell neogenesis can be stimulated in STZ-diabetic newborn rats by administration of the hormone glucagon-like peptide-1 (GLP-1), resulting in improved glucose homeostasis persisting at adult age (Tourrel et al., 2001). In another murine experimental model of alloxan-induced beta-cell destruction, treatment with gastrin and epidermal growth factor (EGF) was found to restore glycemic control and 30–40% of the normal beta-cell mass within 7 days (Rooman & Bouwens, 2004). Combination of the same growth factors proved to be effective also in facilitating islet β-cell neogeneisis in NOD mice with autoimmune diabetes (Suarez-Pinzon et al., 2005). In addition, rescue of endogenous islet function was shown in STZ-diabetic mice after removal of the kidney bearing syngeneic islets, which temporarily maintained mice normoglicemic (Yin et al., 2006), thus indicating that glucose control might be relevant

to facilitate the regenerative process. On the other hand, the possibility that recovery of the endogenous β-cell function may occur independently of glucose control exists, i.e., by the mediation of cytokines, which may activate residual β-cell proliferation or progenitor cell differentiation. To note, in the study by Yin et al., a facilitating role was exerted by the presence of the spleen, which probably plays an indirect role as modulator of the inflammatory process in the pancreas, thereby stimulating recovery of the STZ-damaged islets. STZ seems to trigger a pancreatic regenerative response also in non-human primate models, although it does not lead to a substantial endogenous β-cell recovery in absence of additional stimuli, like the failure of exogenous islet transplantation in the liver (Bottino et al., 2009).

Method	Target Cells	Potential Mechanism for Regeneration
Pancreatectomy	Endocrine Exocrine	Replication of pre-existing β-cells Reactivation of embryonic program
Pancreatic duct ligation	Endocrine Exocrine	Regeneration through Ngn3+ precursors Regeneration through Ca-II and Sox-9+ progenitors
Streptozotocin	β-cell	β-cell neogenesis from ductal cells
Alloxan	β-cell	β-cell neogenesis from ductal cells

CA-II: carbonic anhydrase II

Table 1. Summary of the models used to investigate pancreatic regeneration.

3. Lineage tracing techniques

Lineage tracing techniques have been widely used to investigate both the ontogeny of pancreatic cell fates during mouse embryogenesis as well as the identification of progenitor cells *in vivo* during regeneration (see Figure 2 and 3 for further explanations).

In lineage analysis, specific cells are labeled or marked so that their progeny can be identified later during development. In the pancreas, lineage analysis has been used to recognize not only the progenitor cells giving rise to mature endocrine and exocrine cells, but also the stage at which each set of progenitors is restricted to a particular cell fate. Lineage tracing is also useful to label and isolate marked cells in order to study their gene expression profile and *in vitro* differentiation.

In pancreatic lineage analysis, cells can be labeled using distinct approaches. A physical label - such as dye or a replication-incompetent retrovirus - can be directly injected into embryos to label cells within a tissue. The tissue is allowed to mature *in vivo* or in culture, and the cell types that become labeled reveal the lineage of the starting cells. However, since this method marks cells indiscriminately, in most tissues it cannot be used to label specific sub-populations. A more reliable approach is to genetically mark progenitor cells using endogenous gene expression patterns. This method selectively labels cells that express a particular gene, thus revealing the fate of their progeny. In most cases, a tissue specific promoter (for example Pdx-1) driving Cre recombinase is used to irreversibly tag cells. Other options include the use of a transgene driven by a specific promoter within different cell types, and lineage ablation, either using gene-inactivation mutants (knockout) or transgenic expression of cellular toxins. All these approaches have been used to follow pancreatic cell lineages (reviewed in Gu et al. 2003).

3.1 Cre/LoxP system

Cells can be irreversibly marked using the Cre/LoxP system, thus permitting detection of progeny cells that no longer express the gene of interest. This system uses two transgenic mouse lines, the "reporter" and the "deletor" (Figure 2).

The reporter line uses promoter 1 (Pro.1, black rectangle) to drive reporter gene expression (green rectangle). Upstream of the reporter gene coding region is a STOP cassette made of three repeats of a polyadenilation signal (red rectangle). Flanking the blocking signal are two LoxP sites (blue arrows). Promoter 1 can be tissue specific or ubiquitous. In the deletor line, another tissue specific promoter (Pro. 2, black rectangle) is used to drive the expression of *Cre* recombinase (yellow rectangle). When the two mouse lines are crossed, CRE is expressed in the cells in which promoter 2 is active, thus deleting the blocking signal. This results in the expression of the reporter gene in cells that also express promoter 1.

Fig. 2. Design plan for direct cell lineage analysis.

The first transgenic mouse uses a promoter (promoter 1), which can be tissue specific or ubiquitous, to drive the expression of a reporter gene, such as LacZ or green fluorescent protein (GFP). The second mouse carries a transgene that uses a different tissue specific promoter (promoter 2) to drive the expression of Cre recombinase. In the absence of the Cre deletor transgene, the expression of the reporter protein is prevented by a STOP cassette (multiple repeats of a poly-adenylation signal) upstream of the reporter coding sequence. However, in the presence of Cre recombinase, two LoxP sites flanking the blocking sequence permit this block to be removed. Thus, in double transgenic animals, the reporter gene will be expressed in cells following the excision event, thereby labeling all progeny derived from those precursors that express the deletor transgene.

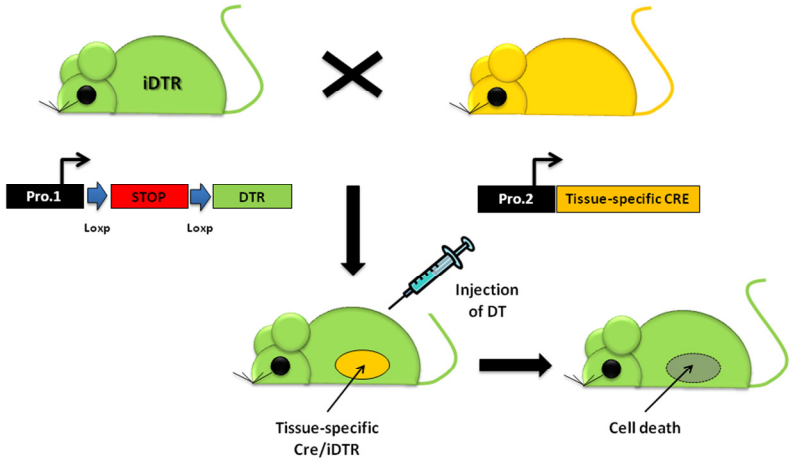
A fine-tuning of the Cre/LoxP system can be achieved with the CRE-ER™ recombinase, which is a fusion between the catalytic domain of the CRE recombinase and the ligand-binding domain of a modified estrogen receptor. The CRE-ER™ protein requires an artificial ligand, tamoxifen, to catalyze LoxP mediated recombination. Because tamoxifen is active within mouse embryos for less than 48 h, cells expressing Cre-ER™ at a specific developmental stage can be selectively labeled by administration of tamoxifen during that stage. After tamoxifen treatment, the conventional CRE recombinase activates the reporter transgene expression as soon as CRE protein is generated, and labeled cells accumulate in any lineage where Cre has been expressed. This type of recombinase can be used to follow selectively the progeny of cells born at defined developmental stages, including postnatal growth and during regeneration.

3.2 Lineage analysis based on simple transgenes

A simpler transgenic approach drives expression of a reporter gene, such as LacZ or green fluorescent protein (GFP), under the promoter of interest. The drawback of this approach is that any progeny of these cells, which cease expression of the chosen protein cannot be followed. In addition, since this method marks cells from the first time the promoter is activated, and as these cells accumulate during development, it becomes impossible to distinguish new members of the population. Thus, one cannot distinguish the progeny of cells born during embryogenesis from those born in adults.

3.3 Lineage analysis based on cell ablation

Another method to investigate lineage relationship is cell ablation. This can be accomplished by specific gene inactivation mutations (knockout), such as in the *Pdx1* knockout mouse, which has no mature pancreatic cells. Alternatively, a tissue specific promoter can be used to drive the expression of a cellular toxin, such as the Diphtheria Toxin A (DTA) subunit. In these transgenic animals, the DTA subunit will kill those cells whose progenitors express that specific transgene. A similar approach is represented by the Diphtheria Toxin Receptor (DTR)-mediated conditional cell ablation model. DTR is a membrane-anchored form of the heparin-binding EGF-like growth factor (HB-EGF precursor). The human and simian HB-EGF precursors bind DT and function as toxin receptors, whereas HB-EGF from mice and rats do not bind the toxin and therefore remain insensitive to DT. Thus, transgenic expression of the simian or human DTR in mice can render cells DT-sensitive. Recently, a mouse strain was generated (iDTR), in which the gene encoding DTR has been introduced into the *ROSA26* locus ($R26^{DTR}$), but its expression is dependent on the Cre-mediated removal of a transcriptional STOP cassette. Therefore, only Cre-expressing cells and their progeny will undergo Cre-recombinase activity and subsequently will transcribe DTR. Although viable and normally functioning, these cells are rapidly killed upon DT administration (Figure 3) (Buch et al. 2003).



The STOP cassette, which prohibits DTR expression, is removed by crossing the iDTR strain to a tissue-specific Cre-expressing mouse strain. Consecutive expression of the DTR renders the respective tissues sensitive to cell death induced by injection of diphtheria toxin. Filled rectangles, *loxP* sites; arrows, transcriptional activity; open ovals, promoter.

Fig. 3. Design plan of the inducible DTR mouse strain (iDTR).

4. Evidence of pancreatic endocrine progenitors/stem cells in the pancreas

Several cells in the pancreas have been described as potential sources of β-cell renewal.

4.1 Pancreatic ductal progenitor cells

During pancreatic organogenesis, stem cells within the duct pancreatic epithelium give rise to both the endocrine and acinar cells (Gittes, 2009). Therefore it seems reasonable to think that the regeneration process could start within the ductal compartment, recapitulating embryonic and fetal development. In addition, there are similarities between islet regeneration and embryonic pancreas development at the gene expression level. Evidence of adult duct cells harboring stem cells capable of differentiating into β-cells was reported both *in vivo* and *in vitro* (Dudek et al., 1991; Ramiya et al., 2000; Bonner-Weir et al., 2000, 2008; Gao et al., 2003). In 2000 Ramiya et al. claimed that long-term cultivation of pancreatic ductal epithelial cells isolated from pre-diabetic, adult, non-obese diabetic mice contained nestin-positive stem cells able to differentiate into islets of Langerhans (Ramiya et al., 2000). These "surrogate" islets responded *in vitro* to glucose challenge, and reversed insulin-dependent diabetes after being implanted into diabetic NOD mice. Similar observations were reported using more defined culture conditions in which isolated human pancreatic duct preparations led to formation and propagation of human islet-like structures (Bonner-Weir et al., 2000, 2008; Gao et al., 2003). Ogata et al. also derived a similar subset of islet-like insulin secreting cells from pancreatic ducts of neonatal rats (Ogata et al., 2004). After incubation with activin A and betacellulin, cells showed tolbutamide- and glucose-responsive insulin secretion. Transplantation of these pseudo-islets in STZ-diabetic NOD mice improved blood glucose levels. Hao et al. confirmed the existence of endocrine stem or progenitor cells within the epithelial compartment of the adult human pancreas, by isolating stem cells from the non-endocrine fraction after islet separation of adult human pancreas digests (Hao et al., 2006). Following elimination of the contaminating mesenchymal cells, the highly purified population of non-endocrine pancreatic epithelial cells (NEPECs) was transplanted under the kidney capsule of immunodeficient (SCID) mice. Although NEPECs produced only low amounts of insulin, when co-transplanted with fetal pancreatic cells, they were capable of endocrine differentiation. No evidence of β-cell replication or cell fusion was observed. To directly test whether ductal cells serve as pancreatic progenitors after birth and give rise to new islets, a transgenic mouse expressing human carbonic anhydrase II (CAII) promoter was generated. This study showed that CAII-expressing cells within the pancreas act as progenitors that give rise to both new islets and acini normally after birth and after injury (PDL) (Inada et al., 2008).

Additional evidence of the existence of endocrine precursor cells within the ductal compartment come from the detection of the nuclear transcription factor Neurogenin-3 (Ngn3) in the ducts during regeneration after STZ. Ngn3 is a basic helix–loop–helix transcription factor, which is able to commit pancreatic cells to an endocrine cell fate (Schwitzgebel et al., 2000). Lack of Ngn3 leads to an absence of islets (Gradwhol et al., 2000); its ectopic expression determines premature over-commission towards the endocrine lineage (Apelqvist et al., 1999). Presence of Ngn3 is very convincing evidence that pancreatic regeneration starts from pancreatic progenitors and mimics the same pathway followed during normal development (Figure 4). By using an inducible Cre-ERTM-LoxP system to

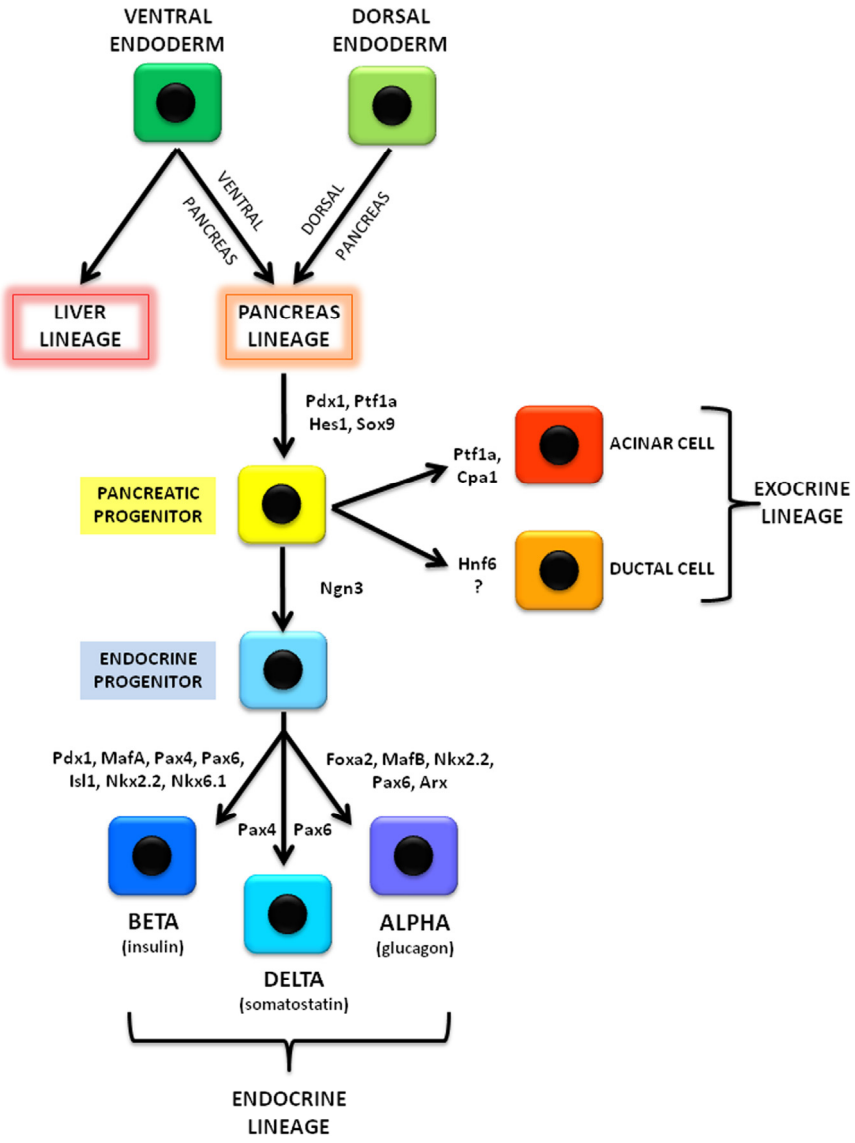


Fig. 4. Regulatory nuclear transcription factors controlling cell type lineages during embryonic pancreas development.

mark the progeny of cells expressing either Ngn3 or Pdx1 at different stages of development, Gu et al. showed that endocrine/exocrine and ductal lineages are separated before E12.5 (Gu et al., 2002). Authors demonstrate that, while cells expressing Pdx1 give rise to all three types of pancreatic tissue (exocrine, endocrine and duct), only the subset Pdx 1/Ngn3^{+} cells are islet progenitors. The duct cells that do not contain progeny of

Ngn3$^+$ cells presumably give rise to the adult duct system and account for the heterogeneity in developmental potential among 'duct-like structures'. Kodama et al. also suggested that in STZ-treated mice, regeneration occurs mainly from intra-islet Ngn3$^+$ progenitor cells rather than from ductal precursors (Kodama et al., 2005). Recently, Xu et al. showed that PDL in the pancreatic tail resulted in some β-cell proliferation and, more strikingly, in a large upregulation of Ngn3 gene expression (Xu et al., 2008). Ngn3 positive cells were found to be closely associated with ducts, and possibly cells of the ductal lineage themselves. After isolation, these cells could give rise to all islet cell types, including glucose responsive β-cells, both *in situ* and when cultured in Ngn3$^{-/-}$ embryonic pancreas explants. In addition, White et al. utilized a system based on Ngn3–enhanced green fluorescent protein knock-in mouse model to isolate endocrine progenitor cells from embryonic pancreata to generate an ample gene expression profile of these progenitors and their immediate descendants (White et al., 2008). On the other hand, a recent publication reported low level expression of Ngn3 in adult endocrine cells, raising concerns about using Ngn3 expression as a marker of endocrine progenitors and neogenesis in the adult pancreas (Wang et al., 2009). Furthermore, another study indicates that although PDL leads to Ngn3 expression in a sub-population of cells within the ducts, it does not induce appropriate cues to allow for completion of the entire β-cell neogenesis program (Kopp et al., 2011).

The potential for the pancreatic organ to recover the endocrine function after injury has been also investigated in non-human primates, where a ductal involvement was observed in STZ-diabetic monkeys that recovered endogenous β-cell function following pig islet transplantation in the liver (Bottino et al., 2009).

Further evidence that hormone-positive cells arise from the ducts comes from the comparison of 16 donor pancreas specimens and pancreas biopsies from 8 simultaneous pancreas/kidney transplantations (Martin-Pagoda et al., 2008). While in the donor pancreas the frequency of insulin$^+$ duct cells was low (0.45%), in five pancreatic transplants with recurrent autoimmunity, 57.5% of the duct cells expressed insulin protein. If new islets were generated from pre-existing ductal tissue, transient co-expression of hormones and residual duct markers could be expected. Indeed, this has been demonstrated in grafts of purified human duct cells (Yatoh et al., 2007).

4.2 Pancreatic non-ductal progenitor cells

Besides ductal progenitors, other groups proposed that pancreatic stem cells may also reside within the islets or in the acinar compartment.

4.2.1 Acinar cells

Acinar cells, which represent the main portion of pancreatic tissue, have been shown to transdifferentiate into islet cells *in vivo* and *in vitro*, through the generation of duct cells as an intermediate step (Lardon et al., 2004; Baeyens et al., 2005; Rooman et al., 2000; Lipsett et al., 2007). Lineage tracing has been used *in vitro* to further strengthen the conclusion that endocrine cells can be generated from exocrine cells via transdifferentiation [Minami et al., 2005]. Earlier *in vivo* lineage tracing experiments in mice showed that acinar cells scarcely contribute to generate new β-cells and duct cells (Desai et al., 2007). However, more recent studies by Collombat et al. demonstrated that upon expression of Pax4, adult α-cells can transdifferentiate to β-cells (Collombat et al., 2009). The ectopic expression of Pax4 forces

endocrine precursor cells as well as mature α-cells, to adopt a β-cell fate. In addition, since α-cells were constantly recruited and converted to β-cells, the resulting glucagon deficiency provoked a compensatory and continuous glucagon$^+$ cell neogenesis through Ngn3$^+$ precursors. On the other hand, Arx misexpression in β-cells, using either an Ins^{Cre} or in adult β-cells using an inducible Pdx1^{CreERT} system reduced insulin-expressing cells and increased alpha and PP-positive cells (Collombat et al., 2007).
Alpha-to-Beta-cell transdifferentiation was also recently described in a transgenic model of diphtheria-toxin-induced acute selective near-total β-cell ablation (Thorel et al., 2010). Lineage-tracing to label the glucagon-producing α-cells before β-cell ablation, tracked large fractions of regenerated β-cells as deriving from α-cells, revealing a previously unknown flexibility in the functioning of the pancreas in relation to hormone secretion, with the potential for exploiting it to cure diabetes.
Generation of β-cells from α-cells has also been shown with a unique model that combines PDL with alloxan-mediated β-cells destruction (Chung et al., 2010). In this model, large numbers of β-cells were generated primarily from α-cells by two mechanisms: the first involved extensive α-cell proliferation, which provided a large pool of precursors that, in turn, would become β-cells via asymmetric division; the second demonstrated that β-cells could form directly from α-cells via transdifferentiation. This latter mechanism was put forward by the finding of intermediate cells co-expressing α- and β-cell-specific markers. Double-positive cells were detectable in the first week after injury, but their number gradually declined and by the second week some converted into mature β-cells, as shown by loss of glucagon and new expression of MafA, a β-cell-specific transcriptional activator.

4.2.2 Nestin-positive cells

Nestin-positive cells have been identified within adult rat islets as being capable of differentiating into insulin-positive cells *in vitro* (Zulewski et al., 2001). Nestin is an intermediate filament protein expressed by the neural lineage, which, according to some groups can be found in the pancreas (Edlund, 2002), in contrast to others that could not find its expression during development of the human pancreatic epithelium (Piper et al., 2002). More recent lineage-tracing experiments showed that nestin-positive cells contribute to the vasculature as well as acinar lineages but not to the endocrine lineage (Treutelaar et al., 2003; Esni et al., 2004; Delacour et al., 2004).

4.2.3 Proliferative human islet precursor cells (hIPCs)

Proliferative human islet precursor cells (hIPCs) were obtained *in vitro* from preparations of adult human islets after extensive *in vitro* proliferation (Gershengorn et al., 2004). Authors believed that these cells, showing a mesenchymal phenotype, derived from insulin-expressing cells undergoing epithelial-to-mesenchymal transition (EMT). hIPCs could be re-differentiated into insulin-expressing islet-like cell aggregates (ICAs) and secreted insulin when transplanted under the kidney capsule of immunodeficient mice**.** However, many criticisms were advanced from other groups, claiming that, at least in mouse pancreatic cultures, islet-derived fibroblast-like cells are not generated via EMT from pancreatic β-cells (Chase et al., 2007; Atouf et al., 2007). Later, Gershengorn et al. further confirmed the basic differences between human and mouse cultures, and claimed that hIPCs are a special kind of pancreatic mesenchymal stromal cells (Morton et al., 2007). More recently, using a lineage-tracing *in vitro* technique, Russ et al. found evidence for massive proliferation of

cells derived from human β-cells. Nevertheless, it appears that induction of significant replication *in vitro* results in dedifferentiation. (Russ et al, 2008).

5. Evidence of pancreatic progenitors/stem cells outside the pancreas

In addition to pancreatic progenitors, cells from other organs, such as liver, spleen, bone marrow, adipose tissue and limbus have been identified as either new sources of islets or stimulators of islet regeneration.

5.1 Liver stem cells

Pancreas and liver share the same origin from the embryonic endoderm (Zaret, 2000). It has been reported that transdifferentiation of pancreas into liver occurs both *in vitro* and *in vivo* in animal models after a number of experimental treatments (Rao et al., 1986, 1995; Dabeva et al., 1997; Kralowski et al., 1999; Shen et al., 2000). The opposite conversion of liver into pancreas is also possible (Horb et al., 2003). Zalzman et al. were able to immortalize a population of human fetal liver epithelial progenitor cells that, once transfected with the Pdx1 gene, generated a stable population of insulin-producing cells (Zalzman et al., 2003). Intraperitoneal transplantation of these cells into immunodeficient mice led to reversal of diabetes for 80 days. However, Yang et al. showed that expression of Pdx1 in hepatocytes does not result in the formation of functional endocrine pancreas in Pdx1 deficient mice, thus suggesting that Pdx1 is necessary but not sufficient to induce differentiation of the pancreatic tissue (Yang et al., 2002).

5.2 Splenic stem cells

The hypothesis that the spleen may harbor stem cells capable of differentiating into β-cells has also been investigated. Faustman and colleagues initially showed that splenocytes contributed to the reversal of autoimmunity in the NOD mouse model when injected with Freund's complete adjuvant (Ryu et al., 2001). Later they also suggested that splenocytes may directly contribute to islet regeneration by differentiation into β-cells (Kodama et al., 2003). However, these findings proved to be controversial. Indeed several other groups, confirming a partial recovery from the autoimmune attack following splenocyte injections, actually failed to display evidence of a direct contribution of donor cells to β-cell regeneration (Suri et al., 2006; Chong et al., 2006; Nishio et al., 2006). Nonetheless, Yin and colleagues supported a facilitating role of the spleen in the regeneration of endogenous β-cell mass (Yin et al., 2006).

5.3 Mesenchymal stem cells

There are numerous reports suggesting that the bone marrow not only harbors haemopoietic stem cells, which are committed to differentiate into blood cells, but also mesenchymal stem cells (MSCs), capable of differentiation into β-cells (Oh et al., 2004; Moriscot et al., 2005). MSCs were reported to differentiate *in vivo* into glucose-competent pancreatic endocrine cells when transplanted in NOD mice (Ianus et al., 2003). However, following studies resulted in controversial outcomes, suggesting that MSCs do not become *per se* insulin producing cells, rather they take part in islet vascularization, eventually promoting β-cell regeneration (Hess et al., 2003 Chamson-Reig et al., 2010). Transplantation of human MSCs was also shown to induce repair of pancreatic islets and renal glomeruli in immunodeficient mice (NOD/SCID) suffering from STZ-induced diabetes (Lee et al., 2006). The role exerted by bone marrow cells

by quenching autoimmunity allowing therefore functional recovery of residual β-cell mass in the pancreas, has been proven by Zorina et al. (Zorina et al., 2003). In the NOD autoimmune diabetes mouse model" in place of, restoration of endogenous β-cell function to physiologically sufficient levels was achievable after allogeneic bone marrow transplantation. Abrogation of autoimmunity and consequent β-cell mass recovery interestingly occurred even when allogeneic bone marrow cell tranplantation was performed after the clinical onset of diabetes. A recent study has suggested that bone marrow cells might have a role in permitting survival of endogenous β-cells also in humans (Voltarelli et al., 2007). Authors reported insulin independence for up to a year in more than half the cases of a small number of patients with recent-onset type 1 diabetes. Patients were administered high-dose immunosuppressive therapy to kill autoreactive T cell clones followed by autologous non-myeloablative stem cell transplantation. Nonetheless, because autologous bone marrow cell transplantation could not change indefinitely the genetic susceptibility to develop autoimmune diabetes, autoimmunity recurred soon after full immunocompetence was re-established. Therefore, different approaches should be used to obtain durable abrogation of β-cell specific autoimmunity, and allow recovery of insulin production (Giannoukakis et al., 2008).

Collectively these series of reports suggest that bone marrow cells do not give rise directly to new insulin producing cells - however they can indirectly facilitate regeneration of the endocrine pancreas, perhaps by secreting appropriate regenerative factors that still need to be characterized.

Other mesenchymal stem cells have been taken into consideration as a potential source of β-cells. Human and rat multipotent adipose tissue-derived stem cells (ADSCs) have been reported to generate insulin-producing cells after transduction with Pdx1 gene. The surrogate β-cells improved glucose sensitivity when transplanted under the renal capsule of STZ-induced diabetic rats [Lin et al., 2009]. In addition, intraportal infusion of human ADSCs together with bone marrow stem cells could increase endogenous insulin levels reducing exogenous insulin requirements in patients affected by type 1 diabetes (Trivedi et al., 2008).

The human limbus has also been indicated as a source of stem cells. The limbus is a highly specialized region of the eye hosting a well-recognized population of epithelial stem cells, which continuously renew the corneal surface. Additionally, the limbal niche also hosts stromal fibroblast-like stem cells (f-LSCs), with multilineage transdifferentiation potential.

f-LSCs were able to generate functional pancreatic hormone-expressing cells *in vitro* recapitulating pancreatic organogenesis (Criscimanna et al., 2011).

6. Evidence of β-cell regeneration via proliferation of pre-existing β-cells

During the fetal stage, differentiation from precursor cells is the major mechanism by which β-cells are formed, while β-cells replication is enhanced during the perinatal and neonatal period. Lineage-tracing experiments in rodents provided convincing proof to the theory that adult β-cells predominantly arise from other β-cells without significant contributions from underlying stem or progenitor cell populations (Cano et al., 2008). Several studies conducted by the group of Melton and colleagues showed that, after pancreatic resection (Dor et al., 2004), or in a diabetic status induced by transgenic expression of diphtheria toxin (Nir et al., 2007), mouse β-cells possess significant capacity for spontaneous regeneration, sufficient to recover from overt diabetes. Authors claim that failure of β-cell regeneration in both autoimmune and pharmacological models of diabetes is due to confounding factors

disguising the innate regenerative response, such as the persistence of circulating autoreactive T cells. To further sustain this hypothesis, it has been demonstrated how therapeutic protocols intended at blocking autoimmunity in NOD mice (Chatenoud et al., 1994) and in humans with type 1 diabetes (Herold et al., 2002, 2005, Bresson et al., 2006) resulted in partial remission from the disease. Whether this is due to a true regeneration process or just recovery of dysfunctional β-cells is still debated.

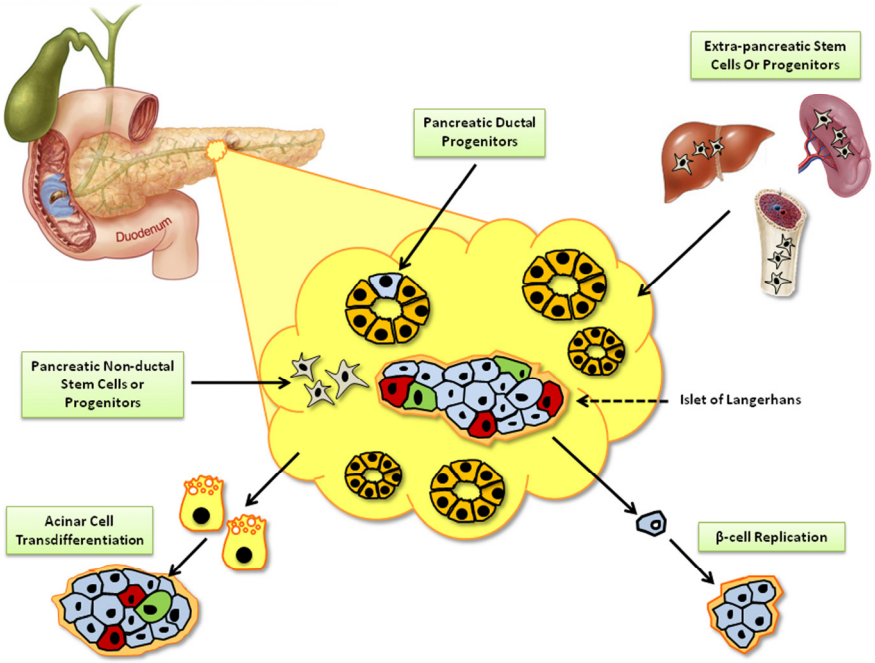


Fig. 5. Schematic illustration of potential cell sources for postnatal ß cell regeneration

Recently, Brennand et al. also demonstrated that in adult mice all β-cells, not just a subpopulation, equally contribute to islet growth and maintenance (Brennand et al., 2007). Two approaches were performed to address this issue. First, evaluation of the replicative potential of the entire β-cell mass was performed by monitoring the disappearance of a fluorescent marker accompanying cell division. Second, clonal analysis of dividing β-cells was completed. Because a uniform loss of label (cell division) across the entire cell population was observed, and all clones were of comparable size, authors conclude that the β-cell pool homogeneously possesses replication ability.

In humans, increased β-cell replication has been documented adjacent to intrapancreatic gastrinomas, suggesting that adult human β-cells can be driven, under specific circumstances, into the cell cycle (Meier et al., 2006b). Support to this remark was also given by another study, which demonstrated that β-cell replication is the primary mechanism responsible for the postnatal expansion of the β-cell mass in a population of young non-diabetic individuals [Meier et al., 2008]. In particular, it was shown that β-cell mass is able to (1) expand by several folds from birth to adulthood, (2) this is accomplished by an increase in number of β-cells per islet with a concomitant expansion in islet size (3) the relative rate

of β-cell growth is higher in infancy and gradually declines thereafter to adulthood with no secondary accelerated growth phase during adolescence, (4) β-cell mass (and presumably growth) is highly variable between individuals, (5) a high rate of β-cell replication is coincident with the major postnatal expansion of β-cell mass. A summary of the theories on β-cell origin is presented in Figure 5.

7. The chronic pancreatitis model

Regenerative responses from the pancreatic tissue can be the result of a surgical or inflammatory injury. It has been described that pancreatic stellate cells, the star shaped cells representing approximately 4% of the resident pancreatic cell pool, are involved in fibrogenesis and pancreas regeneration. In chronic pancreatitis, these cells undergo transformation from quiescent to activated myofibroblast-like cells. Upon activation, which can be triggered by reactive oxygen intermediates, ethanol, Transforming Growth Factor (TGF) α and - β1, they can disclose special features, including the capability to increase the synthesis of collagen, fibronectins, in addition to cytokines. Interestingly TGF-β_1 is expressed by ductal cells in chronic pancreatitis, with a role in development of fibrosis and glandular atrophy (Demois et al. 2002). Recently it has been shown in the adult human pancreas of patients with chronic pancreatitis increased numbers of insulin as well as glucagon -containing cells in the ducts, in addition to other cells containing endocrine and exocrine markers. Such findings were also associated to higher numbers of proliferating cells (Ki67 positive) and $Pdx1^+$ cells, suggestive of a "metaplastic" status (Philips et al.,2007).

In our own experience as a reference center for the isolation of human islet cells, we observed peculiar morphological features in the pancreatic sections in patients with chronic pancreatitis. Not only did we find a higher density of islets, probably due to the destruction of the surrounding exocrine tissue (Figure 6), but, interestingly, we found that the intra-islet β-cell area relative to the α-cell area was significantly higher in chronic pancreatitis patients. Co-expression of the epithelial marker CK19, typical of ductal cells, with Ngn3, a transcription factor expressed in endocrine-committed cells during pancreatic development (Figure 7) was also identified.

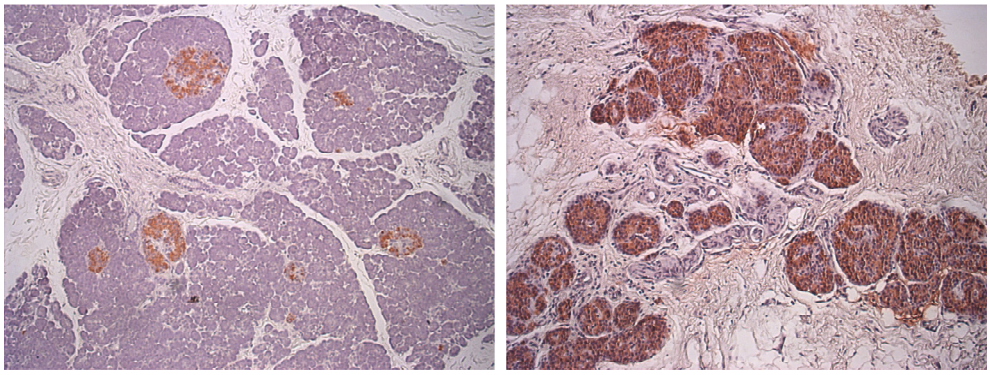


Fig. 6. Histological pancreatic features in chronic pancreatitis.

Islets of Langerhans (immunostained for insulin in brown/red) of a healthy individual (left) in comparison with those of a patient affected by chronic pancreatitis (right).

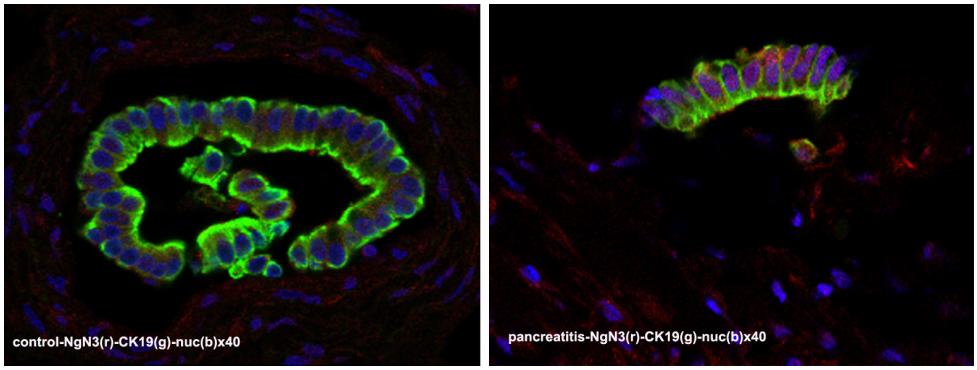


Fig. 7. Peculiar cell phenotypes in chronic pancreatitis.

Co-expression of the epithelial marker CK19 (green) and Ngn3 (red), a transcription factor typically expressed in progenitor cells committed to the endocrine lineage in the pancreas of a patient with chronic pancreatitis (right panel). Control healthy pancreas (left panel).
A number of questions can be asked regarding the process by which damage, and pancreatitis specifically, mobilize these cells. For example, what is the potential role of the immune response to a damage signal in the pancreas in the differentiation of ductal and exocrine cells towards the endocrine lineage? Do immune cells produce soluble factors that facilitate the process of differentiation? What are the characteristics of differentiating non-endocrine and endocrine cells in response to pancreatitis? Can endocrine progenitor cells be better characterized using pancreatitis as an inductive event? Would isolation of such progenitor cells offer a framework for *in vitro* genetic manipulation to further differentiate such cells into defined and fully functional endocrine cells? Would *in vivo* genetic manipulation of the microenvironment exhibiting differentiation processes in response to pancreatitis offer a framework to direct such cells into endocrine lineages? These questions are important in the context of identifying progenitor cells that can serve as the source of endocrine cells, especially insulin-producing β-cells.

8. Conclusions

In conclusion, caution is required in interpreting studies unraveling the mechanisms involved in the maintenance of the β-cell mass. The extrapolation of information from animal studies can be misleading and not necessarily indicative for regenerative therapy in diabetic patients because mechanisms of regeneration/maintenance of the β-cell mass might be different across species (Hanley et al., 2008). For example, in obese rodents, an increment in β-cell mass is achieved by a massive increase of islet size and β-cell number per islet, consistent with a predominant mechanism of β-cell replication. On the contrary, in obese humans, islets are only modestly increased in size, consistent with minor involvement of β-cell replication (Butler et al., 2003). Also, a 90% pancreatectomy performed in young rodents leads to transient diabetes and regeneration of β-cell mass of ~50% within 2 weeks (Bonner-Weir et al., 1983, 1994), while only a 50% pancreatectomy is sufficient to determine diabetic status in adult humans who afterward became obese too. Certainly, one potential explanation for the reported differences between rodents and humans could be that rodents are more frequently studied at 1–3 months of age, when there is a high capacity for β-cell

replication, whereas human pancreatic tissue is primarily studied in adults. It is therefore of great value to investigate the potential of pancreatic tissue to recover endogenous endocrine function after injury in animal models more similar to humans, such as non-human primates. However, even if the mechanisms regulating the maintenance of the β-cell mass in physiologic conditions are the same in humans, monkeys and rodents, we cannot exclude that during pathological conditions different pathways, species-specific and injury-specific (for type and magnitude), might be activated. For example, although the potential of acinar, duct (epithelial) and mesenchymal cells to differentiate into other cell types has been demonstrated *in vitro*, the mechanisms *in vivo* may be totally different. These discrepancies would not be surprising as cells taken into a foreign environment commonly behave differently than when residing in their natural niche. Another consideration is that, if regeneration occurs by recapitulation of fetal development, adult precursors should respond to fetal inductive signals. Conversely, if regeneration occurs by a different pathway, it is less likely that the process would be influenced by the same signals. The study of the factor(s) secreted by bone marrow precursors may shed some light on these aspects of the regenerative process as well.

In conclusion, the debate between the supporters of neogenesis and self-renewal for maintenance and replacement of β-cells is still open. It is perhaps acceptable to conclude that there is some truth in both proposed hypothesis, and that one does not exclude the other. Understanding the potential contribution of each mechanism will be crucial to find better curative approaches for diabetes.

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18

Cell Replacement Therapy: The Rationale for Encapsulated Porcine Islet Transplantation

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1. Introduction

Among the problems posed by chronic diseases today, one of the most daunting is that posed by diabetes mellitus, which is a very significant public health problem resulting in substantial morbidity and mortality (American Diabetes Association, 2011). With the increasing life-expectancy of the world's population, increasing exposure to environmental trigger factors, the rising incidence of obesity, and lifestyle changes such as unhealthy diets and decreased physical activity, the prevalence of diabetes has risen dramatically over recent years and is now reaching epidemic proportions globally. This rapid increase is a significant cause for concern, with an additional 7 million diagnosed each year (International Diabetes Federation, 2011). Diabetes is now the fourth or fifth leading cause of death in most developed countries (International Diabetes Federation 2000). Globally, it is estimated that more than 200 million adults now have diabetes and this number is expected to increase alarmingly in the coming decades. By the year 2025, it is estimated that almost 333 million people will have the disease (International Diabetes Federation 2006).

Type 1 (insulin-dependent) diabetes accounts for 5% to 10% of all diagnosed cases. In 2003, approximately 4.9 million people (0.09% of the world's population) were estimated to have type 1 disease, with Europe having the highest number of sufferers (1.27 million) followed by North America (1.04 million) and Southeast Asia (0.91 million). The highest prevalence of type 1 diabetes was in North America (0.25%) followed by Europe (0.19%) (International Diabetes Federation 2006). In 2002, there were an estimated 0.9 to 1.2 million people with type 1 diabetes in the USA (American Diabetes Association 2006). The incidence in recent years may have accelerated alarmingly as shown in a recent study from Finland where the rate increased from 31.4 per 100,000 per year in 1980 to 64.2 per 100,000 per year in 2005 (Harjutsalo et al, 2008).

In New Zealand, the incidence of type 1 diabetes has doubled in the last 15 years, reflecting international trends. In 2003, the estimated prevalence of type 1 disease among the population aged <25 years was 0.18%, with the total number of sufferers in this age range numbering 2540. The majority, 85% (2158 people), were of European descent, while 9% were Maori, 2.9% were Pacific peoples, and 3.0% were Asian (Wu et al. 2005).

Although the life-expectancy of patients with type 1 (insulin-dependent) diabetes mellitus has vastly improved since the introduction of insulin, the ability of insulin injections to

reliably prevent wide fluctuations in blood glucose levels is often inadequate and many patients develop complications of the disease. These complications cause considerable disability and suffering, and their management has major morbidity and cost consequences. High blood glucose concentrations not only cause acute metabolic problems but also lasting and accumulative damage by chemical reaction (glycation, e.g. Haemoglobin A1c) with a host of physiologically critical proteins. Hence the long term damage to the cardiovascular system, eye, kidney, heart and nervous system. On the other hand, low blood glucose levels, known as hypoglycaemic episodes, are usually perceived by the patient and treated by ingesting glucose or food. But in a significant minority of patients, hypoglycaemic episodes are not perceived and may cause loss of consciousness. These can lead to fatal outcomes for the patient and sometimes for others, such as in situations where the patient is in control of a moving vehicle.

For these reasons, the investment in, and search for, newer treatments that can provide a 'cure' for the disease with normoglycaemic control, or that at least minimizes the damaging effects of extremely high and low blood glucose excursions with markedly better control of metabolic disturbances, has been energetically pursued. In terms of health economics, the benefits in preventing eye, heart and kidney diseases would more than justify a substantial investment in such research and development (Beckwith et al. 2010).

1.1 Background and rationale for cell transplantation

Insulin is essential for normal glucose metabolism. It is released by the beta-islet cells of the pancreas in response to rising blood glucose levels. The feedback mechanisms involved provide a precise, finely tuned response, keeping blood glucose at a concentration of around 4.5mM. Type 1 diabetes is an endocrine disorder caused by autoimmune destruction of the beta-islet cells leading to insulin deficiency. Treatment by injecting various commercially produced insulins subcutaneously, while life-sustaining, can not provide the control of blood glucose provided by a full complement of functional islets.

In order to optimize the control of blood sugar and thus prevent the acute and chronic damage, the most likely way to improve these outcomes is to replace the patient's pancreatic islets with a new pancreas or new islets. Transplanting a whole new pancreas is a very demanding procedure that requires many resources and is found to be less than practical.

1.1.1 Porcine islet cell transplants

Among the newer treatment strategies that have been proposed, transplantation of pancreatic islets, obtained either from other human or animal donors, has received considerable attention worldwide. This is because islet transplantation can restore not only the insulin-secreting unit, but also the precise insulin release in response to rising blood glucose and multiple signals arising within and beyond the islets.

Because human islet transplantation is limited by the shortage of human islet tissue, human embryonic stem cells or induced pluripotent stem (iPS) cells from the patient are being developed into transplantable insulin producing cells. Recent reports indicate that iPS cells may not be transplantable into mice of the same strain without immune rejection (Zhao et al, 2011). The US FDA guidelines highlight concerns that stem cell derived lines may undergo malignant transformation or develop into teratomas after transplantation (Fink et al 2009).

While stem cell line-derived insulin producing cells are still at an early stage of research, pig islets are viewed as a promising alternative since: (a) the supply of pig pancreatic cells can be

increased by breeding more donor animals; (b) pig and human insulins have close structural and biological similarities; and (c) physiological glucose levels in pigs are similar to those in humans (Elliott, 2011). The rationale for this treatment approach (termed 'xenotransplantation') is that the implanted pig islets have the potential to mimic the normal physiological insulin response in type 1 diabetics, such that near-normal blood glucose levels may be achievable without insulin injections or with reduced requirements. As a consequence, long-term diabetes complications may be prevented and patients should experience less hypoglycaemia than they do with the currently recommended 'intensive' insulin regimens. Thus the need and rationale for improved diabetes control is clear but the effectiveness and practicality of islet transplants, whether from human, porcine or other sources, has yet to be firmly established. As with any transplanted tissue, organ or cells, whether from human or animal, the host immune system must be considered. Immunosuppression has been studied and used extensively in islet transplant patients. An increasingly successful alternative is immune-isolation, that is to isolate the transplanted cells in capsules or devices that exclude immune cells and antibodies, but allow the free diffusion of glucose, insulin, nutrients and dissolved oxygen and carbon dioxide. The latter approach eliminates the risks associated with immunosuppression.
Some of the **key issues** in implementing **porcine islet xenotransplantation** in the treatment of patients with Type 1 diabetes are:

1.1.1.1 Have 'proof of concept' pre-clinical experiments demonstrated sufficiently effective improvement in the control of Type 1 diabetes in experimental animals?

1.1.1.2 Have the risks to the patient's safety been sufficiently evaluated, including: (a) the risk of transmission of infectious diseases; and (b) risks to the individual from the transplant procedure itself, including allergic reactions and other immunological responses that might compromise the success of the procedure.

1.1.1.3 How rejection of the cells by the recipient's immune system can be effectively prevented.

1.1.1.4 Whether porcine islet xenotransplants can restore, at least partially, the normal regulation of blood glucose (as reflected in decreased insulin requirements and decrease in HbA1c), and the number of islets needed to achieve this.

1.1.1.5 The duration of effectiveness of the transplanted islets (i.e. whether they remain effective over a sufficiently prolonged period to justify the inconvenience and cost of the procedure), and the extent to which the patient's well-being is enhanced and long-term diabetes complications are prevented.

1.1.1.6 Ethical considerations, including cultural, ethical and spiritual dimensions, informed consent issues, and measures to ensure animal welfare.

1.1.1.7 If clinical trials are judged successful by rigorous independent review, will resources to expand the availability of the treatment be provided by commercial and/or government investment?

2. Pre-clinical studies of porcine islet xenotransplantation in non-human primates and rodents

The main body of information from our laboratory on the efficacy and safety of current preparations of porcine islets is derived from studies with islets sourced from Auckland

Islands Pigs. The Auckland Islands (AI) strain is unique due to its isolation for more than 150 years on a sub-antarctic island and is free of pathogens commonly found in other pig herds. These animals are being bred in custom-constructed pathogen-free facilities for transplant purposes and are discussed in detail later in this chapter.

2.1 Studies in non-human primates

The first exploration of primate diabetes treatments with encapsulated porcine islets was undertaken by Sun Y et al (1996) in Toronto, Canada. This pioneering group prepared islets and enclosed them in alginate-polylysine capsules in the early 1980s (Sun AM et al, 1984; Sun Y et al, 1993). They were able to find a number of diabetic cynomolgus monkeys that were ideal for testing these encapsulated porcine islets. These animals responded very well to their encapsulated porcine islets transplant, with significant improvement of their diabetes for 120-800 days. This remarkable achievement was based on decades of research by AM Sun, Y Sun and their colleagues that began in the early 1970s.

In a later non-human primate study the clinical efficacy and safety of an encapsulated neonatal porcine islet preparation was investigated in cynomolgus monkeys with streptozotocin (STZ)-induced diabetes. The islet toxin streptozotocin is injected into the monkeys and causes insulin deficiency. Sixteen monkeys with the disease established were separated into two groups of 8: one group was given microcapsules containing living porcine islets and the other was given the same microcapsules but without islets in them. They received the microcapsule transplants in two doses three months apart by injection into the peritoneal cavity (Elliott et al, 2005). No immunosuppressive drugs were administered.

In the group that received capsules containing islets, evidence of clinical activity was noted at 12 and 24 weeks after the first transplant; the reduction of the mean weekly insulin requirement relative to the control was 36% ($p=.02$) and 43% ($p=.01$), respectively at these time points. Blood glucose in the two groups were maintained to similar levels indicating that the reduced requirement for insulin injections in the treated group was significant. Both the islet-treated and control groups tolerated the transplant procedures well. No hypoglycaemic episodes or other adverse events were observed in the islet-treated group. There were no differences between the two groups of monkeys in body weight or hematology and liver enzyme parameters. Two deaths occurred, one in the islet-treated group from lobar pneumonia with disseminated lung abscesses at 13 weeks after the first transplant (despite systemic antibiotic therapy), and the other in the control (empty microcapsules) group as a result of a stroke that followed the development of hypoglycaemia due to failure to consume food after regular insulin therapy.

At terminal autopsy of these monkeys (Figure 1), no gross inflammatory reactions to either encapsulated islets or the empty microcapsules were noted in the animals' peritoneal cavities. The organs (liver, spleen, stomach, intestines, kidney, heart, lungs and brain) appeared normal .

In another streptozotocin diabetic monkey study, where encapsulated adult porcine islets were used, and thus more similar to the adult human islet isolations, diabetes was successfully treated without immunosuppression (Dufrane et al, 2010). However, although efficacy was demonstrated with encapsulated islets, they lost their function after 2 weeks. Successful glucose control was achieved for 6 months using 'monolayer cellular devices' containing up to 30,000 Islet Equivalents (IEQ)/Kg of monkey weight, implanted in the abdominal subcutaneous tissue.

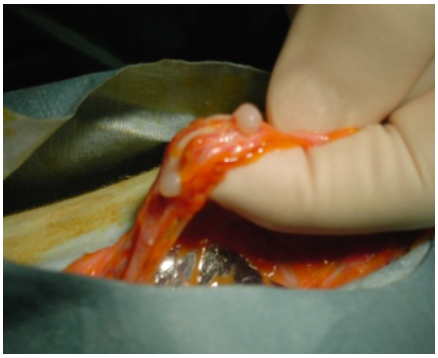

Fig. 1. Clusters of encapsulated porcine islets attached to the mesentery of a cynomolgus monkey.

In contrast, Hering et al (2006) demonstrated that unencapsulated naked porcine islets, injected into the portal vein of the liver, could be used to reverse diabetes for 100 days in cynomolgus monkeys provided comprehensive immunosuppression was given.

2.2 Studies in small animal models of diabetes

The biocompatibility of encapsulated porcine islets and a dose-dependent effect on glycaemic control have been demonstrated in STZ-diabetic rats. Those that developed diabetes were separated into four groups that received intraperitoneal transplants of encapsulated neonatal porcine islets at increasing dosage. The doses, measured in IEQs, were 3000 IEQ (n = 7), 6000 IEQ (n = 6), 12,000 IEQ (n = 6) or 18,000 IEQ (n = 6) of the standard preparation or empty alginate microcapsules (n = 12), insulin requirements were significantly reduced in rats given the higher doses. Prior to transplantation, all rats had comparable blood glucose control with daily isophane insulin injections. At week 12 after transplantation, the reduction in the weekly average daily insulin dose was significantly greater in rats given doses of 18,000 IEQs ($p < 0.01$) or 12,000 IEQs ($p < 0.05$) compared with control animals that received empty alginate microcapsules. Insulin independence was attained in 3 of 6 rats given 18,000 IEQs, 3 of 6 given 12,000 IEQs, 2 of 6 given 6000 IEQs. 1 of the 12 diabetic rats given empty alginate microcapsules did recover, a case of spontaneous islet regeneration which is a known confounding factor in small animal studies (Figure 2).

Similar encouraging results were seen when streptozotocin-induced diabetic mice and genetically predisposed diabetic NOD mice were treated with encapsulated islets.

Thus, immunosuppression may not be necessary when treating diabetes if the islets are efficiently sealed inside capsules or devices with semipermeable membranes. The capsule or device outer membranes exclude immune cells and immunoglobulins but must allow efficient diffusion of glucose, insulin, all nutrients and gases (oxygen, CO_2) necessary for cell nutrition.

This information about the efficacy of encapsulated islets and their tolerance by animal recipients, including some earlier human clinical studies, were assembled in great detail for the submission of applications to the National Regulatory Clinical Trial Agencies of several countries including New Zealand, Russia and Argentina, where the applications were successful, albeit with stringent conditions attached.

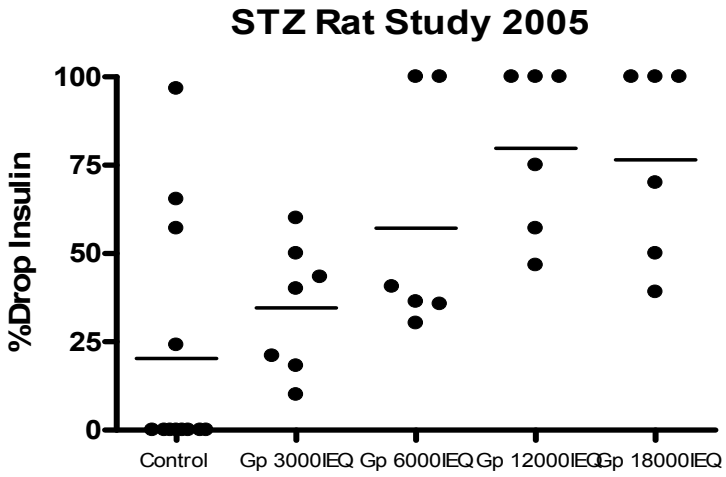

Fig. 2. **Percentage reductions in weekly averages of daily insulin doses 12 weeks after transplantation.** Each point represents individual rats. Reduction of the insulin dose is calculated as the percentage of the baseline average daily insulin dose in the week prior to transplantation. In the three higher doses eight animals were insulin-independent at 12 weeks. The bars represent the average % reduction in insulin dose for the respective groups.

3. Xenotransplantation: Minimizing risks to patient's safety

The introduction of porcine islet transplantation has been delayed by concerns relating to the possible transmission of pig diseases to humans via the transplanted cells and the risk of introducing micro-organisms during cell processing and encapsulation. There is also a potential risk of transmitting porcine disease to close contacts of the human recipient and to the wider community. The risks of infection from the donor animal can be minimized by controlling the breed, source of and health status of the donor animals, with ongoing screening and quarantining. The risk of introducing micro-organisms during cell processing can be minimized by ensuring strict aseptic technique when isolating and encapsulating the islets.

3.1 Risk of infections ('xenoses') resulting from transfer of pig micro-organisms to human recipients

The risk of bacterial, fungal and parasitic infections can be minimized by the use of Designated Pathogen-Free pig herds and by monitoring and treating sows for such infections before pancreatic islets are extracted from their progeny (Garkavenko et al. 2008a). However, this approach will not eliminate porcine endogenous retrovirus sequences (PERVs), which are present in the germline of all pigs (including common New Zealand pig breeds) but cause no known infection in the species. The possible transmission of these retroviruses to humans, and their as yet unknown consequences in recipients, have given rise to some concerns over the safety of xenotransplantation, particularly in view of reports that PERVs from certain pig strains can infect human cells in vitro (Patience et al. 1997;

Martin et al. 1998a) and that immune-incompetent SCID (severe combined immunodeficiency) mice may develop either microchimerism or infection in vivo (van der Laan et al. 2000). The in vitro findings have, however, been shown to be strain-specific (Patience 2001; Clemenceau et al. 2001; Oldmixon et al. 2002) and cells from animals studied by LCT have not shown retrovirus infectivity. Moreover, no evidence of PERV transmission has been detected over 200 patients who have been exposed to pig cells or tissues and tested for evidence of PERV infection using sensitive detection methods (Wynyard et al. 2011; Garkavenko et al. 2008b; Denner 2003; Dinsmore et al. 2000; Heneine et al. 1998; Heneine et al. 2001; Irgang et al. 2003; Martin et al. 1998b; Paradis et al. 1999; Patience et al. 1998; Tacke et al. 2001). In 2 New Zealand diabetic patients who received encapsulated porcine islet xenotransplants, no evidence of PERV proviral DNA or RNA was detectable in white blood cells and plasma up to 6 years after the transplant, and neither patient was found to have suffered any ill health as a result of the procedure (Elliott et al. 2000; Garkavenko et al. 2004a). Similarly, in 4 other patients who received unencapsulated islets in similar studies in NZ, no infection has been found in a follow-up time of up to 9 years (Garkavenko et al. 2004a). More recently one patient shown to have some functional transplanted porcine islets after 9.5 years was shown to be free of PERV.

3.2 Porcine endogenous retrovirus

In several other studies, no evidence of PERV transmission was found among recipients of porcine clotting factor VIII (Hyate:C) in a study of 88 haemophiliacs, despite the fact that all manufactured batches of porcine factor VIII concentrate used by patients were subsequently tested and shown to contain PERV RNA (Heneine et al. 2001). Similarly, no evidence of PERV DNA was found in 2 renal dialysis patients whose circulation had been linked extracorporeally to pig kidneys (Patience et al. 1998).

3.3 Auckland Islands pigs

A further refinement of the source herd has been obtained by the use of pigs derived from a colony abandoned on the Auckland Islands about 150 years ago. These animals are free of all measured viruses except the retrovirus (PERV), and are being bred for xenotransplant purposes in a purpose-built facility. Although PERV cannot be removed from the porcine genome, there is strong evidence that it is not functional in the Auckland Island Pig strain and is not expressed under any of the recommended testing conditions. This strain is now termed a "Null Pig Strain" suitable as a source of tissues for xenotransplantation (Wynyard et al, 2011).

3.4 Neonatal pigs

Nevertheless, the selection of donor animals that do not transmit infectious PERV continues to be important and has to be linked with the selection of donor animals bred in isolation and screened to exclude infection with other exogenous microbes. The use of donor neonatal piglets rather than adult pigs also has the advantage of limiting the exposure time of donor newborn animals to acquired infections as they age.

3.5 No genetic modification

The selection of piglets without genetic modification as a source of tissue offers another advantage. In attempting to prevent immune rejection of porcine tissue, genetically-modified

pigs with the gene for the xeno-antigen alpha-gal eliminated have been developed. However, with the use of alpha-gal gene knock-out pig donor cells, PERV-exiting cells are not enveloped (coated) with the alpha-gal antigen and hence are not recognised as 'foreign' by the recipient's blood complement system (Fujita et al. 2003). It is known that normal human serum contains natural anti-alpha-gal antibodies that inactivate retrovirus (Rother et al. 1995).

3.6 Intact immune system

The maintenance of an intact immune system is an important safety factor. Immunosuppressive drugs are commonly used to prevent immune rejection of transplanted organs and cells. However, the use of alginate-encapsulated islets is intended to allow the survival of transplanted islets and their continued secretion of insulin in the recipient, without the need for life-long immunosuppressive drugs.

3.7 Surveillance

Long-term surveillance of recipients of porcine islet xenotransplants and testing of the transplant material for the presence of PERVs, using highly specific and highly sensitive assays developed for this purpose will always be an integral part of strategies proposed for future clinical trials. This safeguard, which is a necessary precaution recommended for clinical trials of xenotransplantation by authorities in various countries including the USA (US Department of Health & Human Services 2002, 2003), Australia (National Health & Medical Research Council 2002), UK (United Kingdom Xenotransplantation Interim Regulatory Authority 1998, 1999), and Canada (Therapeutic Products Programme, Health Protection Branch, Health Canada 1999), is designed to allow early detection of infectious disease transmission, and includes standard hospital infection control measures to limit the spread of such an infection should one be detected. These carefully structured and independently monitored precautionary measures are now mandatory for all patients enrolled in clinical trials of porcine islet (or other cells) xeno-transplantation and are put in place before the trials begin.

4. Minimizing risks of contaminating with infective agents during product preparation

After the pancreas is removed from the donor animal it must be treated with a series of procedures to isolate the insulin-producing islets, substantially free of the proteolytic exocrine tissue which makes up the majority of the pancreas. It is then kept in cell culture medium and tested for microbiological contamination and for its ability to produce insulin in response to stimulation by exposure to a glucose challenge. This must be done with every batch of islets produced for clinical use. The manufacturing facility, records and staff training need to be regularly inspected by expert teams from government health agencies. Samples of islets from every batch and biopsies of pancreas, heart, lung, spleen, kidney and brain tissues, also blood samples, from every donor animal must also be stored frozen at -80 Centigrade as historical resources that can be retrospectively analyzed, in case of later complications in patients.

5. Preventing rejection of islets by the recipient's immune system

The vulnerability of transplanted islets to the recipient's immune system has been a major scientific challenge and a barrier to successful islet transplantation. The transplanted cells

face not only Immediate Blood Mediated Immune Rejection (IBMIR) but also immune attack from a variety of cells which may result in loss of function and cell death. Two approaches are currently used to overcome this:

5.1 Immunosuppressive drugs

The administration of immunosuppressive drugs before a cell transplant and for the rest of the life of the patient. These procedures to manage the immune competence of the patient are essential in greater or lesser forms for all transplants of naked cells except perhaps those that are sourced from the patient (autotransplantation) or from a perfectly matched donor. Even then, any patient-derived cells (e.g. endogenous adult stem cells) that may be induced to mature into functional islet cells may be attacked by auto-immune processes engendered during the auto-immune destruction of the patient's original pancreatic islets, since the newly matured islet cells carry essentially the same antigens (Zhao et al, 2011).

A suppressed immune system, with lower surveillance of foreign antigens, can provide an opportunity for infection. Immunosuppressive agents may also prevent the essential immune surveillance that detects and destroys most cellular chance mutations that lead to cancer cell growth. This has been a serious concern about stem cell transplants that may contain a small number of viable undifferentiated cells that can exhibit uncontrolled replication and form tumours (Bauer SR, 2010).

5.2 Immunoisolation

Immunoprotection of the transplanted cells via the use of a semipermeable membrane that acts as a protective barrier (Figures 3a and 3b). The latter technique appears to be a viable approach, the principle being that the permeability of the outer capsule membrane allows smaller molecules such as glucose, dissolved oxygen and carbon dioxide and all nutrients to penetrate into the capsule and reach the islets. The membrane is constructed to allow insulin and most small proteins to be released out into the bloodstream, but it does not allow the passage of large immune cells or antibodies that would cause rejection of the islets. Indeed, the islets can survive well inside such systems. There are several approaches to providing this immunoprotection which are:

- Encapsulation of the islets within a bead of alginate gel, which is then coated with poly-L-ornithine, poly-L-lysine or some other material to provide perm-selectivity and strength (Calafiore 2006; Weir & Bonner-Weir 1997).
- Tubular diffusion chambers which consist of long (up to 20mm) tubular membranes of 5-6mm inner diameter.
- Perfusion devices (also known as vascularised artificial pancreases) which consist of an outer housing 90 mm in diameter and 20 mm in width that is connected surgically to the patient's vascular system so that it is fed by an artery and drained by a vein. Islets in both perfusion devices and diffusion chambers are usually immobilised with alginate or agar to prevent settling and to provide uniform distribution of nutrients and dissolved oxygen and carbob-dioxide (Maki et al. 1995).
- Co-transplantation with 'nursery' cells such as testicular Sertoli cells which have been claimed to protect against immune-mediated rejection via the production of the immunomodulator TGF-beta1 (transforming growth factor-beta 1) (Suarez-Pinzon et al. 2000; Valdes-Gonzalez, 2005).

Of these approaches, the strategy selected by Living Cell Technologies for intensive investigation is encapsulation of the islets in 'minimal volume' alginate microcapsules developed at the Department of Internal Medicine and Endocrine & Metabolic Services, University of Perugia in Italy (Calafiore 1997; Calafiore et al. 1999). Alginate-encapsulated porcine islets have been extensively investigated in experimental animals both with and without diabetes, and in a small number of human diabetic subjects.

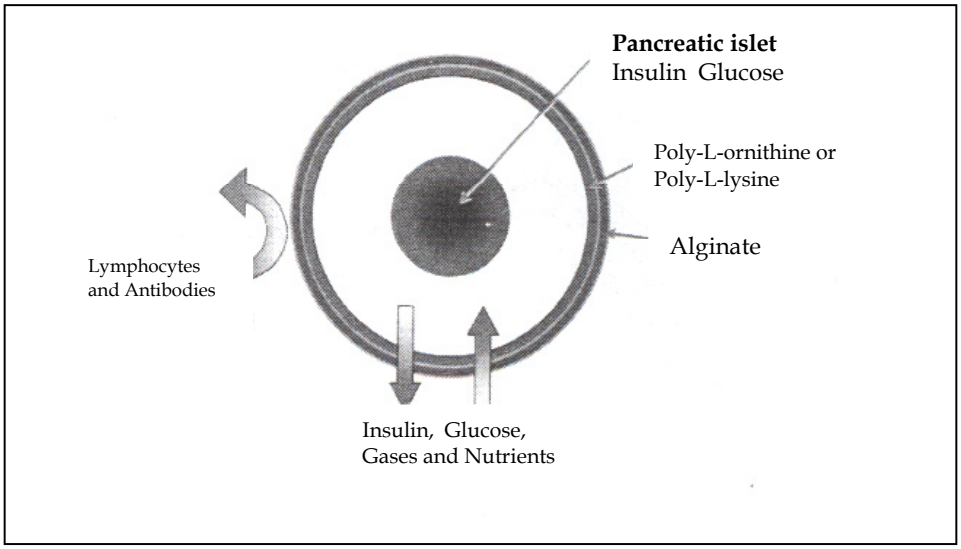

Fig. 3a. **The concept of encapsulation:** enclosure of the islet in an alginate/poly-L-ornithine or poly-L-lysine membrane that is permeable to glucose, nutrients and insulin, but not to lymphocytes and antibodies, provides protection against immune destruction of the cells.

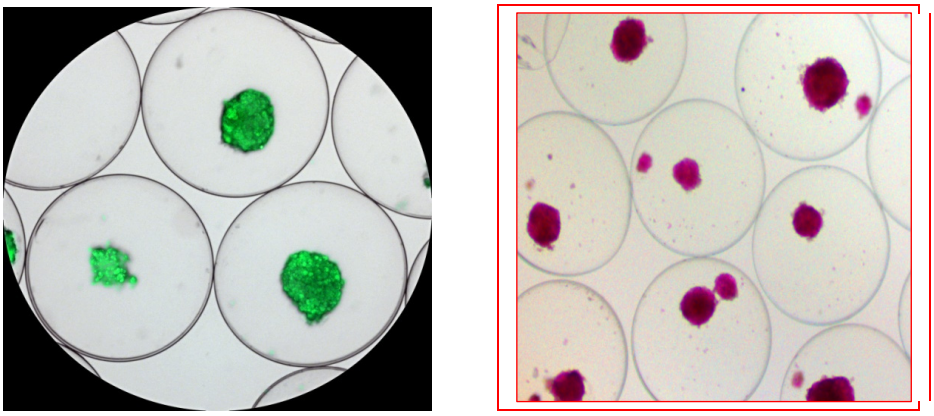

Fig. 3b. **Islets isolated from neonatal porcine pancreas in alginate-polyornithine capsules.** Green fluorescent stain (acridine orange) demonstrating viable cells and red tetra-methyl-rhodamine-ethyl ester (TMRE) stain for mitochodria of viable cells.

6. Alginate-poly-l-ornithine capsules

These capsules, as shown in **Figure 3**, have been the method of choice for many laboratories including our own (Opara & Kendall, 2002; Hernandez et al, 2010; Elliott, 2011). Their biocompatibility is critical to their in vivo efficacy and durability. Alginate is derived from seaweed and has to be highly purified to remove contaminating heavy metals, proteins and endotoxins. Different sources of alginate vary in their chemical composition with different ratios of their main constituents of mannuronic acid and guluronic acids and hence their physical gel properties. The consistency of the microcapsules with respect to their size, wall thickness, compressibility and cell occupancy has to be ensured from batch to batch. The central core containing the islets is liquefied alginate and the outer surface is a membrane formed by ionic interaction between positively charged poly-L-ornithine (or poly-L-lysine) and negatively charged alginate. Properly made, the membrane is remarkably robust (Skinner et al, 2009).

Since up to a million capsules may be required for treatment, the production must be reproducible, using durable equipment to Good Manufacturing Practice (GMP) standards. The main components are carefully engineered flow-through needles which generate reproducible sized droplets impelled by air 'knife' flows or electrostatic mechanisms (Hernandez et al, 2010).

7. Ethical considerations

Embodied in the wider concepts of informed consent are considerations that young people and the old or infirm are not exploited. Peoples with poor understanding of the language and scientific concepts involved are not to be misled. Religious and cultural feelings are not to be disregarded in potential recipients who find it difficult to express their reluctance to participate in the presence of those whom they perceive as medical authorities.

The ethical issues of xenotransplantation include concerns over the cultural, ethical and spiritual dimensions of xenotransplantation; the ethical acceptability of using animals to provide tissue for human transplantation; how the welfare of donor animals can be adequately protected and their suffering reduced; and how the welfare and interests of patients in early clinical xenotransplantation trials can be protected. These issues have been addressed by the Nuffield Council on Bioethics in the United Kingdom (1996) and, in New Zealand, the Bioethics Council, Toi te Taiao (2005). The latter body concluded that prohibition of xenotransplantation could not be justified, given the compelling human need argument, and that it should be allowed to develop in New Zealand, with that development being demonstrably shaped by the resolution and management of safety issues by a competent authority; the relationship between the majority European culture and indigenous Maori people and the cultural, ethical and spiritual factors that matter to most New Zealanders.

In recent years, a number of recommendations to protect the ethical integrity of future human research have been made by various regulatory authorities. Key issues in the conduct of clinical trials of xenotransplantation include the requirement to provide patients with an explanation of the likely success, its attendant risks, and the subsequent quality-of-life that can be expected when obtaining their informed consent, and informing patients that their consent to the procedure includes consent to ongoing post-transplantation microbiological monitoring.

Other international spiritual organisations have given their opinions about xenotransplantation. The Vatican in Rome, Italy have considered xenotransplantation in some depth and suggest it is worthy of serious consideration (Vatican, 2011). Jewish organisations have given qualified support to xenotransplantation as a life enhancing procedure while Muslim opinion is unclear but has generally been negative because of the status of pigs in the religious context.

8. Clinical experiences with xenotransplantation in treating type 1 diabetes

Many of the considerations in the preceding part of this chapter have been opinion and experiment seeking to determine the safety and efficacy of xenotransplantation for Type 1 diabetes in animal models. These animal models are perhaps more or less imperfect as true reflections of the human condition of Type 1 diabetes. It is therefore of critical relevance to review all data and experiences relating to the small number of human exposures to xenotransplanted porcine islets and other cells.

Six patients were treated in 1995-6 with either encapsulated or unencapsulated neonatal porcine islets. One of these, a 41-yr-old Caucasian male with type 1 diabetes for 18 years was given an intraperitoneal transplant of alginate-encapsulated porcine islets at the dose of 15,000 islet equivalents (IEQs)/kg bodyweight (total dose 1,305,000 IEQs) via laparoscopy. By 12 weeks following the transplant, his insulin dose was significantly reduced by 30% ($p = 0.0001$). The insulin dose returned to the pre-transplant level at week 49. Improvement in glycaemic control continued as reflected by total glycated haemoglobin of 7.8% at 14 months from a pre-transplant level of 9.3%. Urinary porcine C-peptide, derived from the porcine pro-insulin precursor, peaked at 4 months (9.5 ng/ml) and remained detectable for 11 months (0.6 ng/ml). The patient was followed as part of a long-term microbiologic monitoring program which subsequently showed no evidence of porcine viral or retroviral infection.

The patient opted for elective laparoscopy 9.5 yr after transplantation. Abundant nodules were seen throughout the peritoneum. Biopsies of the nodules showed they contained capsules still protecting living cell clusters. Immunohistology noted sparse insulin and moderate glucagon staining cells. The retrieved capsules produced a small amount of insulin when placed in high glucose concentrations in vitro. An oral glucose tolerance test induced a small rise in serum of immuno-reactive insulin, identified as porcine by reversed phase high pressure liquid chromatography (Elliott et al, 2007).

With this demonstration it was clear that this form of xenotransplantation treatment has the potential for sustained benefit in human type 1 diabetics.

Since then two further human studies have started using porcine islets in microcapsules and without immunosuppressive drugs. In 2007, a pilot study with 8 patients was approved by the Scientific and Ethics Committees of the Sklifosovsky Institute, Moscow where islets were obtained from biocertified designated pathogen free pigs and encapsulated under GMP conditions in New Zealand. Adult patients were aged 23-63 with type 1 diabetes as defined by the American Diabetes Association criteria. They were insulin dependent for 5 -15 years. Before the implants, patients had to have stimulated plasma c-peptide levels < 0.2 ng/ml to confirm insulin deficiency. Their diabetes had to be inadequately controlled with HbA1c of > 7% pre-implant. Patients were administered 5,000 or 10,000 islet equivalents per kilogram body weight (IEQ/kg). There were no significant adverse events and no evidence of zoonosis. Patients were also given repeat implants with no untoward effects. Preliminary

data shows a reduction in daily insulin dose and reduction in HbA1c compared with pre-implant values following the first implant (Tables 1 and 2) in the majority of patients. Two patients became insulin independent for a period, the maximum being 32 weeks. At the repeat implant, 6 months after the first implant, intact microcapsules were retrieved and subsequently found to contain viable cells. Porcine insulin was also detected in the circulation following glucagon stimulation.

Patient ID	Dose 1st Tx (IEQ/kg)	Insulin Dose	% Dose Reduction	
		Pre 1st Tx	Post-1st Tx	
			3-month	6-month
1	5,000	113	32	34
2	5,000	22	100	32
3	5,000	60	-7	5
4	5,000	30	13	13
5	5,000	68	15	16
6	10,000	41	?	0
7	10,000	37	73	100
8	10,000	83	0	0
Mean		57	32	25

Table 1. **Reduction in daily insulin dose after first implant (Tx) in 8-patient pilot study.** Patient 2 was insulin-independent at 3 months but subsequently needed insulin support. Patient 7 became insulin-independent at 6 months.

Patient ID	Dose 1st Tx (IEQ/kg)	HbA1c (%)		
		Pre-1st Tx	Post-1st Tx	
			3-month	6-month
1	5,000	7.1	6.3	6.9
2	5,000	8.2	7.3	7.0
3	5,000	10.0	7.8	7.3
4	5,000	7.6	7.9	7.6
5	5,000	9.8	6.2	7.2
6	10,000	8.5	-	-
7	10,000	8.3	4.9	6.5
8	10,000	11.3	8.2	8.6
	Mean:	8.9	6.9	7.3

Table 2. Reduction in Haemoglobin A1c (HbA1c) after first implant (Tx) in 8-patient pilot study.

In 2009, a Phase I/IIa study approved by the New Zealand government following international peer review and Ethics Committee Approval was commenced. It will include 14 adult patients. Unlike the preceding clinical studies, these patients were required to have unstable type 1 diabetes accompanied with hypoglycemic episodes. The patients are to be administered a single intra-abdominal implant of 5,000, 10,000, 15,000 or 20,000 IEQ/kg. To date no significant adverse events have been attributable to the treatment. At this early stage of the open label study, episodes of hypoglycemic unawareness (Figure 4) and hypoglycemic convulsions have been eliminated in the first patient. This was associated with significant reduction in the severity of hypoglycemic scores. A full one year follow-up of all patients is expected to be completed at the end of 2011. A third trial will be conducted in Argentina in 2011/2. The clinical studies are thus still at the stage of dose finding to determine the optimum dose and dosing regimen.

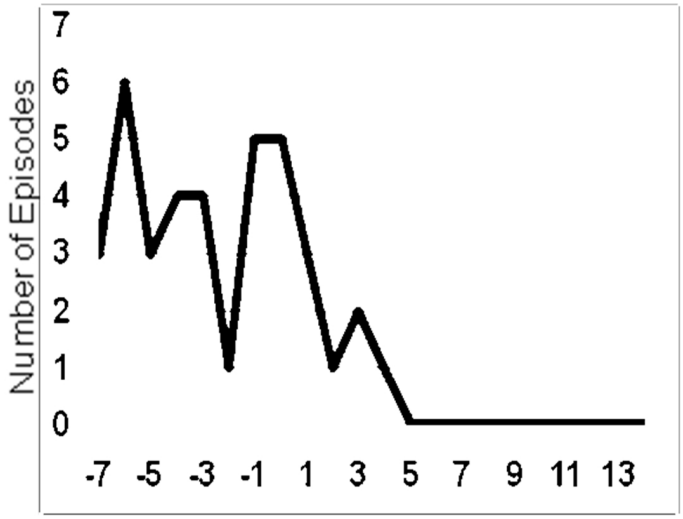

Fig. 4. Example of Elimination of Episodes of Unaware Hypoglycemia (Patient #1, Phase I/IIa Auckland, New Zealand)

9. Conclusion

Xenotransplantation is not yet standard treatment. There is current research into the feasibility of using donor animal organs such a kidney and liver. However, unlike cells, organs are difficult to screen against potential infectious agents. If clinical trials with cell transplants for diabetes are successful, porcine islet transplants will lead the way for the use of other cells for the treatment of nervous system disorders and enzyme deficiencies (Skinner et al, 2009).

Porcine islet xenotransplantation has the potential to be beneficial for those with absolute insulin deficiency. There is now a consensus that the procedure is relatively safe from xenotic infections. The results of several non-human primate and rodent studies indicate significant efficacy may be achieved. The early results of human clinical trials also suggest

that this form of treatment for Type 1 diabetes, without immunosuppression, is worthwhile. There is the concern that the treatment may be too expensive to be a practical treatment for the large numbers of patients who are expected to benefit from porcine islet implants. However, a health economic analysis of quality adjusted life years suggests that this approach may be cost effective taking into account the current cost of treating the disease and complications of cardiovascular disease, blindness, limb amputation, end-stage kidney disease and neuropathy (Beckwith et al, 2010).

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Part 5

Diabetes and Oral Health

19

Dental Conditions and Periodontal Disease in Adolescents with Type 1 Diabetes Mellitus

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1. Introduction

The importance of type 1 (diabetes mellitus) DM lies in the function it plays in dentition and oral health for children and adolescents. The consequences of DM concerning oral health are in connection with the systemic changes[1] caused by the disease, though the results are in certain cases conflicting. Oral manifestations related to DM may have a strong inclination to periodontal disease[23], as well as an increased incidence of dental caries, mucosal lesions and dry mouth (xerostomia). However, as mentioned before, the results are not entirely in accord[456]. Children and adolescents with type 1 DM are more susceptible to infections in the dental connective tissues than those without type 1 DM. The dentist's liablility is to evaluate the dangers and maintain adequate oral hygiene to prevent the occurrence of disfunctional oral effects of type 1 DM.

2. Periodontal disease

Periodontitis is a chronic multifactorial plaque induced Gram-negative anaerobic infection of the periodontium that results in the destruction of periodontal tissues and loss of alveolar bone.[7] Periodontitis is to be treated by the mechanical removal of supra- and subgingival bacterial plaque with scalers, curettes or ultrasonic devices (scaling and root planing [SRP]), and by instructing the parient about oral hygiene. The development of new dental plaque deposits and re-infection of the subgingival tissues can only be prevented in case of a near-ideal oral hygiene. For the improvement of clinical periodontal status the regular use of systemic or local antibiotics as an adjunctive therapy to SRP is still concerned problematic.[8910] Deep residual periodontal lesions can usually be reduced or eliminated by surgery.

2.1 Diabetes and periodontal disease

There is an extensive debate on the influence of DM on the risk of periodontal disease. According to most of the experts, patients suffering from diabetes mellitus are highly susceptible to develop periodontal disease.[111213141516] In 1993, periodontal disease was termed as the sixth complication of diabetes[17], while in the report of the Expert Comittee on the Diagnosis and Classification of Diabetes Mellitus issued in 1997, periodontal disease was mentioned as a disease of very high incidence in case of patients with diabetes.[18] Many papers on the subject have outlined that the inclination, extent, severity and development of

periodontal disease are significantly increased in case of patients with diabetes.[19][20] Impaired immune response, different bacterial microflora and collagen metabolism are involved in the pathogenesis of diabetic periodontal disease[21]. This is especially true if the diabetes is poorly controlled[22] and there is a lasting periodontal disease in case of type 1 DM individuals[23].

Gingival bleeding is a sign of inflammation. Vascular changes in diabetes mellitus may result in increased gingival bleeding. If however the diabetes is adequately treated, there are fewer vascular changes.[24] Gingival bleeding is in positive correlation with the accumulation of plaque and calculus.[25] Diabetics showed more plaque and higher calculus index scores than the control groups.[26] Calcium concentration was high in both the parotid and submandibular saliva of patients with type 1 DM.[27] This result may explain the regularly experienced increase in calculus formation in the case of these patients.[28][29] Individuals with a decreased glucose tolerance showed a higher rate of pocket formation, presence of calculus, increased tooth mobility and tooth loss.[30] Variable models have been developed to give reason to this correlation such as periodontal chronic inflammation which results in increased circulating cytokines and inflammatory mediators, autoimmune response to the chronic periodontal infection that gives way to endothelial dysfunction, or the presence of certain factors that leads to increased inclination both to periodontal disease and diabetes vascular diseases at the same time.[31]

Statistically significant relation has been detected between the HbA1C levels of the patients and the biomass, haemolysin activity and proteinase activity. The biofilm formation is likely to have an effect on the pathogenicity of oral candidosis.[32]

There is, however, a minority of experts who are sceptical about the high risk of type 1 DM.[33][34][35][36]

Today Glickman's theory is the most widely accepted one. According to Glickman, the father of modern periodontology, diabetes are not the direct cause, but a predisposing condition for periodontal disease.[37] There is a bidirectional relationship between diabetes and periodontal diseases[38]. Diabetic adolescents have more prevalence and more severe gingival inflammation than healthy control persons of the same age[39], despite similar plaque scores[51]. Metabolic imbalances in the tissues may reduce the resistance of diabetics to infection,[40] and thus affect the initiation, developement and progression of periodontal disease.[41] Adolescents with type 1 DM develop an earlier and higher gingival inflammatory response to a similar bacterial challenge than non diabetics.[34][42] In accordance with this fact, extended studies have shown that severe periodontal disease in diabetic patients is primarily related to poor metabolic control and other diabetes complications occurring during the follow-up process.[43][44] Still, long-duration diabetics resulted in more severe periodontitis than short-duration diabetics.[45] It has been proposed that fine periodontal treatment of diabetic patients may lead to the lowering of diabetic complications.[46][47][48] Chronic Gram-negative infections and chronic endotoxaemia, which arise in case of periodontal disease, may also increase insulin resistance and reduce metabolic control in case of diabetic patients. This entails the hypothesis that elimination or control of periodontal infection may improve the metabolic control of diabetes.[49][50] It has been proposed that a microbiological imbalance in the gut may increase the gram-negative bacterial load, which would also increase the systematic inflammatory burden through the lipopolysaccharides leakage into the circulation. Increased inflammation leads to insulin resistance.[51][52][53] According to a study, insulin requirements were lowered following periodontal treatment and reduction of inflammation in diabetic patients.[54] It has been demonstrated that the interaction of periodontal bacterial byproducts with mononuclear phagocytic cells and fibroblasts induces the chronic release of cytokines (IL-1β, IL-6, TNF-α), PGE2 and CRP.[55]

Recently many studies have proposed that periodontal disease is a substantial aggravating factor in restoring the health of patients with diabetes.[56] It is mainly due to the fact that it maintains a chronic systemic inflammatory process. However, the causality between periodontitis and altered glycaemic control can only be justified epidemiologically with prospective interventional studies.[57] The control of periodontal disease could be an essential part of the overall treatment of diabetic patients: the periodontal therapy may improve glycaemic control, and could lead to a significant lowering of HbA1c values.[40]

Concerning the adolescents, the oral hygiene seems to be better in case of the diabetic patients than in case of the other group of young patients who have shown highly variable work shifts leading to poor oral hygiene. The difference is reflected by a lesser bacterial plaque among the diabetics. This is in conflict with the results of other authors who observed similar oral hygiene status and plaque indexes in the two groups, or even comparatively increased plaque accumulation among the diabetics.[58] The reason for this may be the following: although the diabetics were controlled, they showed a high gingival response to the irrigation caused by bacterial plaque retention.[59] Regularly diabetics have a greater incidence regarding periodontal disease than healthy individuals[6061], though other studies have not detected such relation between periodontal disease and diabetes.[626364] They didn't confirm any association between diabetes and periodontal disease in adolescents. They believe that there is no difference between on the one part type 1 DM adolescents and children, and on the other healthy individuals regarding clinical periodontal status. However Fructosamine value, used to diagnose and monitor diabetes, was observed to be in correlation with the gingival index score in case of type 1 DM adolescents and children, but not in case of healthy control subjects.[65] Bay et al.[34] found no difference in the degree resolution of gingivitis following scaling and root planning between young diabetics and healthy control group. Barnett et al.[35] studied periodontitis prevalence in young diabetics but none of the individuals showed radiographic sign of periodontitis.

Despite the comparatively better control of bacterial plaque among the diabetics, a change in their periodontal response was detected. Therefore it seems that local immune response may possibly change among these patients, and the lesions caused by microbial agents in case of periodontal disease, which can be associated with a lesser tissue repair capacity, might be the reason for the increased deterioration of periodontal structures shown in the diabetic group.[4]

It is possible, that the diversities observed by the different authors result from local health care habits. In an area with a tradition of good oral hygene, the oral hygiene habits of diabetes are not better than those of healthy people. Therefore in case of similar oral hygiene habits, diabetical patients show lower periodontal values than healthy persons because of the above mentioned immune response. In areas where the dental treatment habits of the population are poorer, the greater attention of well-controlled DM patients to oral hygiene results in higher values than those of healthy individuals.

2.2 Prevalence of gingivitis, periodontitis in adolescents with type 1 DM

Since there are conflicting reports in the literature, the aim of this study was analyse the periodontal disease in adolescents with type 1 diabetes mellitus.[66] Characterization of risk factors, local such as oral hygiene-, general (duration of DM, age, degree of metabolic control of DM) and contributory factors-, such as toothbrushing, have been identified that determining the risk factors place people enhanced risk in development and progression of periodontal diseases.

A dental clinical cross-sectional examination was carried out on 259 adolescents of ages 14-19 with type 1 DM in comparison with a non-DM group as control group. Children who had been under the age of eight at the onset of DM were excluded, as where those with any additional disease or taking other chronic medication. The control group comprised of metabollically healthy individuals. The DM patients were classified according to the categories recommended by the World Health Organisation (1999). The Greene-Vermillion OHI-S index was used to determine the level of oral hygiene. Periodontal changes were rated by the Russell's periodontal index (PI), which estimates the degree of periodontal disease occurring in the mouth by measuring both bone loss around the teeth and gingival inflammation. This method is regularly used in the epidemiological investigation of the disease.[67] Alveolar bone loss was tested according to Schei et al (1959), and Hirschman et al (1994) with panoramic radiograph and vertical or posterior bitewings radiographs.

The DM subjects were characterised according to the following criteria: mean postprandial blood glucose level during the period of 6 months prior to the dental examination; glycosylated haemoglobin (HbA1C) level; duration of the DM; the age at the onset of DM. The ADA (2005) criteria were used concerning the DM control. Diabetes mellitus was well-controlled (in 210 cases), if six months before the dental examination the mean postprandial blood glucose level of the patients was normal or near normal (below 7.5 mmol/l), there was no glycosuria HbA1C≤ 6.5%, and severe hypoglicaemia did not occur. Control was taken to be poor when the postprandial blood sugar was ≥ 7.5 mmol/l and/or haemoglobin was > 6.5%, or in case of patients with asymptomatic hypoglicaema.

The incidence of gingivitis and periodontitis was much higher in case of diabetic adolescents than in case of metabolically healthy persons ($p< 0.0001$) and especially in case of girls ($p< 0.001$). Healthy periodontium was only found in case of 2.6% of the DM adolescents and the rest of DM patients had either gingivitis (61.03%) or periodontitis (39%), while a high rate of metabolically healthy persons (80.5%) has shown a healthy periodontium. Gingivitis and periodontitis was experienced in case of 18.4% and 1.1% of the patients. (Fig.1.)

With the fall of oral hygiene (OHI-S) index ($p< 0.0001$) the intensity of gingivitis and periodontitis (PI) has become more severe. (Fig.2.)

An important positive correlation has been shown between the control level of the disease and the intensity of gingivitis and periodontitis. In case of well-controlled 'Type 1' diabetic adolescents the PI mean value was lower than in case of patients with poor glycemic control ($p< 0.001$).

In the diabetic group the periodontal disease was detected to be severe in case of those who had a lasting DM (>5 years) ($p<0.001$).

The majority of the control persons showed no alveolar bone loss (83% had intact alveolar bone) and those who had alveolar bone loss, had primarily that of horizontal type. In case of diabetic adolescents prevalence of intact alveolar bone was lower (61.8%) than in case of the control group. Therefore higher degree of alveolar bone resorption (both horizontal and horizontal+vertical type) was experienced in case of diabetic patients than in case of the control group ($p<0.001$).

The mean values of alveolar bone resorption were higher in case of diabetic boys than in case of diabetic girls ($p<0.0001$).

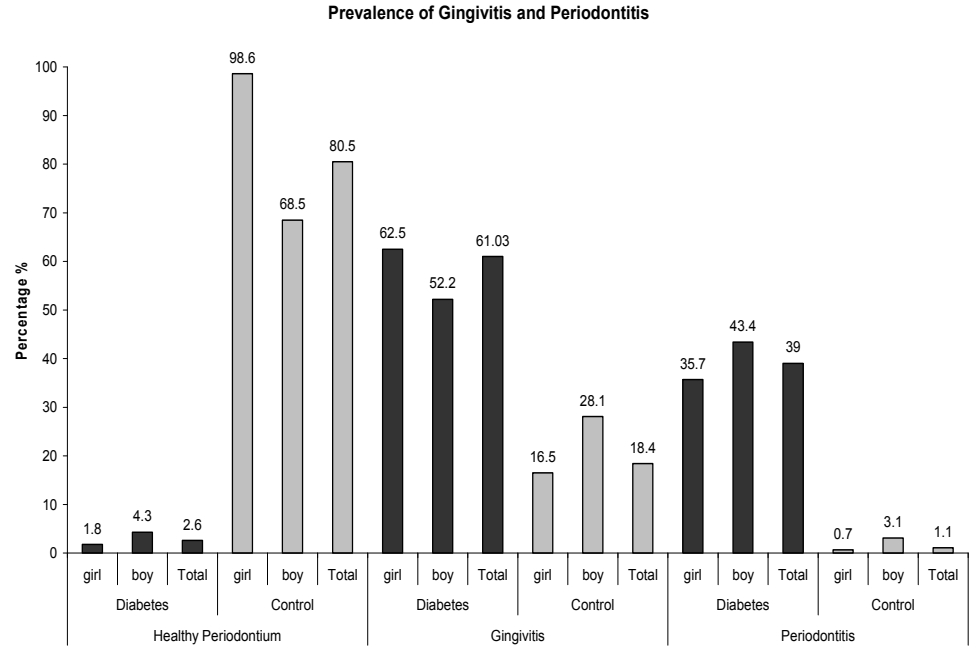

Fig. 1. Prevalence of gingivitis and periodontis

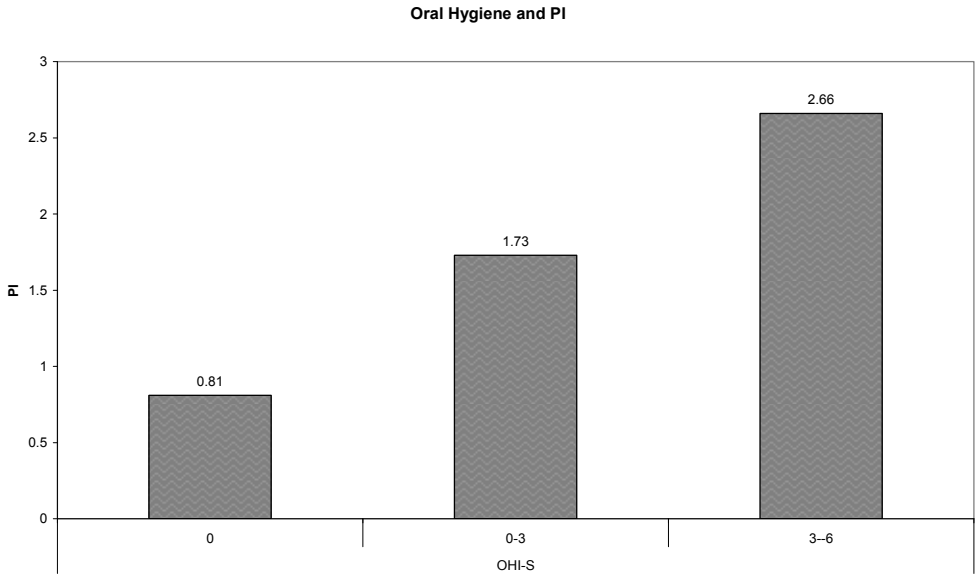

Fig. 2. The mean value of PI according to oral hygiene (OHI-S)

The intensity of gingivitis and periodontitis, as well as the increased alveolare bone loss was more prevalent and more severe in diabetic adolescents than in healthy individuals. Several authors suggested a correlation between diabetes mellitus and periodontal diseases. According to them periodontal disease is one of the most prevalent and rapidly progressive complications[3738] of DM in case of adults.

Sappala et al. (1993) has shown that adults with Type 1 diabetes mellitus have higher degree of attachment loss and bone loss than control subjects under similar dental plaque conditions. The authors received the same results for diabetic adolescents. The oral hygiene (OHI-S), especially the debris index was worse in case of diabetic individuals than in case of the control persons and correlation was found between on the one part the intensity of gingivitis and periodontitis and on the other the oral hygiene. However De Pommereu et al.[57] found more serious gingivitis in diabetics than in control persons, although plaque scores were the same in both groups. Interestingly, according to the present study diabetic adolescents, especially boys have more severe alveolare bone destruction than non-diabetics.

The present study demonstrates that severity of periodontal disease increases with the duration of diabetes mellitus, in agreement with the results in case of adults measured by Pavez[23] and Greene Vermillion (1964), Rosenthal et al. (1988), and Lopey et al. (2002).

This study shows that the poorly controlled type 1 DM with elevated blood glucose and glycosylated hemoglobin (HgA_{1c}) levels have more incidental and serious periodontal diseases with alveolare bone loss in case of adolescents with lasting diabetes mellitus. Adequate metabolic control of diabetes mellitus lowers inclination to infection. This is also crucial for the prevention of periodontal disease in case of adolescents with diabetes mellitus. In case of adult diabetic patients with poorly controlled diabetes mellitus, increased metabolic control may improve periodontal condition (Miller et al. 1992, Yki-Jarvinen 1989, Harris 1995, RST/AAP 1999).

Periodontitis is a complex multifactorial disease. It can especially cause a problem for adolescents with DM. Inadequate oral hygiene is responsible for high oral debris in case of diabetic adolescents, being in correlation with the status of periodontium.

Several other charcteristics such as long duration, early onset (under 14 years), and degree of metabolic control, have been determined as factors increasing the risk of disease. In case of diabetic girls the monthly hormonal level alterations of the gingival may play a role in the development and progression of gingivitis, periodontitis and alveolare bone resorption.

Frequent dental treatments may help maintain good oral health. Treatment is especially crucial at the onset of the disease. Dental care at the early stages of the disease, reducing susceptibility to infection, is also significant for the prevention of periodontal disease in case of adolescents with type 1 diabetes mellitus. Children should also be checked regularly for bleeding gums or inflammation for to prevent the alveolare bone loss, which leads to irreversible changes of periodontium.

The education of youngsters on proper home oral care is the basic method for periodontal greatment and prevention. In most cases the gingivitis can be resolved by plaque control.

3. Caries

Dental caries is a chronical bacterial disorder where bacterial processes damage hard tooth structure (enamel, dentin, and cementum).[68] The tissues break down progressively, resulting in dental caries (cavities, holes in the teeth). The basic factors are the causal microorganism, the host (tooth), the substrate (diet), and the immune capacity of the patient.

Two groups of bacteria may cause caries: *Streptococcus mutans*[69] and *Lactobacillus*[2570]. Bacteria gather around the teeth and gum in a sticky, creamy-coloured mass called plaque, which serves as a biofilm. Bacteria in an individual's mouth transform glucose, fructose, and most regularly sucrose into acids such as lactic acid through a glycolytic process called fermentation.[71] In direct relation with the tooth, these acids may lead to demineralization, i.e. the dissolution of its mineral content. However the process is dynamic. If the acid is neutralized by saliva or mouthwash, remineralization may arise. Fluoride toothpaste or dental varnish can help remineralization.[72] If the demineralization process is continuous, enough mineral content could be lost so that the soft organic material left behind disintegrates, forming a cavity or hole. Caries is one of the most common diseases of the world at present.

3.1 Diabetes and caries

Epidemiological studies do not seem to be in accord on the characteristics of diabetes mellitus (DM) concerning the incidence of dental caries in case of both children and adults[737475767778]. Although adolescents may be more caries-prevalent than individuals of other ages, only few studies deal with this age group. In case of type 1 diabetics an increased prevalence was detected[79], located particularly in the root or dental neck regions.[70] However studies differ whether control of DM or sucrose-free diet of DM patients is more likely to promote or inhibit the development of dental caries. Opinions do not match concerning the dental condition for patients with DM or the outcome of early DM manifestation regarding the dental condition.

Cross-sectional studies have reported a low prevalence of caries in case of children and adolescents with type 1 DM, and this has mainly been explained by the sucrose-restricted diet, which is a part of the lifelong treatment.[8081] According to Wegner,[82] the frequency of caries in DM childen is at least not lower than in non-DM children. Wegner[83] also observed that, directly after the diagnosed onset of their disease, some young DM patients displayed a higher activity of caries than healthy subjects of the same age, but the frequency of caries gradually diminished in association with dietary restriction and treatment with insulin. Other researchers proposed a relationship between the development of caries and the level of metabolic control.[298485] These researches showed a higher prevalence of caries in case of poorly controlled DM than in case of well-controlled disease. The reason is that higher glucose content in oral fluids adds to bacterial proliferation, enhancing the formation of dental plaque. It has been shown for all concerning age groups that children with DM have less caries than non-diabetic children.[26] However, these experiments also demonstrated that the result was due to an abundance of sites where teeth were lost without replacement. The complications were not symptoms of type 1 DM, they might be caused by poor medical and dental care. Some studies proposed that salivary secretion rates should be substantially lowered in case of children with type 1 DM when compared to healthy children.[86] Reduced salivary secretion increases the probability of caries, although adequate metabolic control prevents the most dangerous salivary alterations such as high glucose content and low pH, while a fine diabetic diet, rich in fiber and poor in simple carbohydrates, can decelerate the development of plaque and the proliferation of acidogenic bacterial microflora.[2987] Individuals at ages of adolescence generally clean their teeth less frequently than persons at ages after adolescence.[88] Behavioral factors such as dental self-efficacy are in relation with DM self-efficacy and adherence.[71] Adequate oral health habits may lead to lower incidence of caries. On the other hand, Moore et al. observed similar regularities concerning the use of dentifrice and dental floss between diabetics and non-diabetics[89]. However other studies demonsrtated that diabetic patients used dental floss more frequently than non-diabetics.[90] It seems that there are contradicting studies on the dental condition in case of patients with DM as well as on the effect of early DM manifestation on the dental condition.

3.2 Dental caries in adolescent with type 1 DM

Since there are conflicting reports regarding the dental condition in patients with DM or the effect of early DM manifestation on dental condition, the aim was to analyse the dental status DMF-T and the prevalence of dental caries in adolescents with type 1 DM and compare the findings with those from metabolically healthy individuals, in an effort to determine the risk factors play a role in the development of dental caries in DM adolescents.[91]

A dental clinical cross-sectional examination was carried out on 259 adolescents aged 14-19 years with type 1 DM in comparison with a non-DM group as control. Children who had been under the age of eight at he onset of DM were excluded, as where those with any additional disease or taking other chronic medication. The control group comprised metabollically healthy individuals. The DM patients were classified according to the categories recommended by the World Health Organisation (WHO). Caries was assessed by the DMF-T index. Within this index, F was used for the filled teeth, D denoted the number of untreated carious teeth without regard to whether the lesion was enamel or of root. Extractions for orthodontics or periodontal reasons were excluded. The Greene-Vermillion OHI-S index was used to determine the level of oral hygiene. The DM subjects were characterised according to the following criteria: mean postprandial blood glucose level and during the period of 6 months prior to the dental examination; glycosylated haemoglobin (HbA1C) level; the duration of the DM; and the age at the onset of DM: Concerns by DM control, the ADA (2005) criteria were offered. Diabetes mellitus was well-controlled (210 cases) if six months before the dental examination the mean postprandial blood glucose level of the patients was normal or near normal (below 7.5 mmol/l) there was no glycosuria HbA1C≤ 6.5% and severe hypoglicaemia is did not occur. Control was taken as poor when the postprandial blood sugar was ≥ 7.5 mmol/l and/ or haemoglobin was > 6.5%, or in cases of patients with asymptomatic hypoglicaema.

The DM adolescents had a slightly higher mean DMF-T score than the control subjects. The difference was found to be statistically significant ($p<0.001$).

When the components of DMFT were considered, there were more filled (F) ($p<0.001$) and fewer decayed teeth (D) ($p<0.0001$) among the DM adolescents than in the healthy controls. When the number of missing teeth was considered, there were no significant differences between the DM patients and the healthy controls.

The DM boys had a slightly higher mean DMFT score than the DM girls ($p<0.001$). More decayed (D) ($p<0.001$) and filled (F) teeth were found among the DM boys than among the DM girls ($p<0.001$). As regard the number of extracted (M) teeth, there were no significant differences between the boys and the girls.

The age of the patient at the onset of DM was correlated to the caries condition (D). This suggested that the early onset of DM (before the age of 14) was related to significantly fewer decayed and filled teeth compared with the patients in whom the DM had developed after age 14 years ($p< 0.01$). (Fig.3.)

However the adolescents with good oral hygiene (OHI-S=0), there was a significant differences ($P<0.0001$). (Fig.4.) A positive correlation was found between of the level of control of the DM and the dental condition. In the well-controlled DM adolescents the mean number of decayed (D) teeth was lower ($p< 0.0001$), but the numbert of filled (F) teeth was higher than in patients with poorer glycemic control($P<0.001$). (Fig.5.)

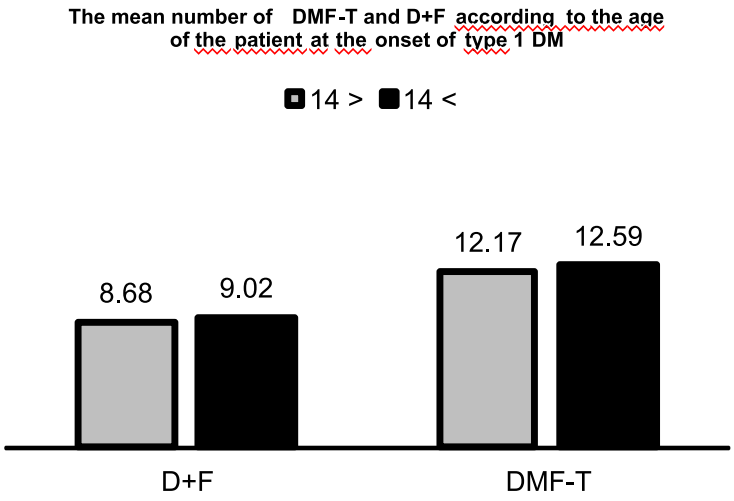

Fig. 3. The mean number of the DMF-T and D+F according to the age of the patient at the onset of type 1 DM

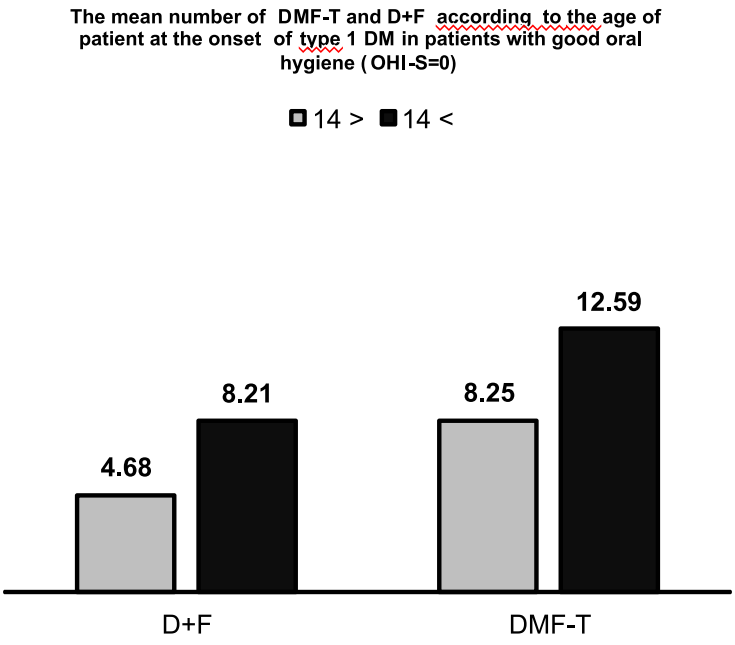

Fig. 4. The mean number of the DMF-T and D+F according to the age of the patient at the onset of type 1 DM in patients with good oral hygiene (OHI-S=0)

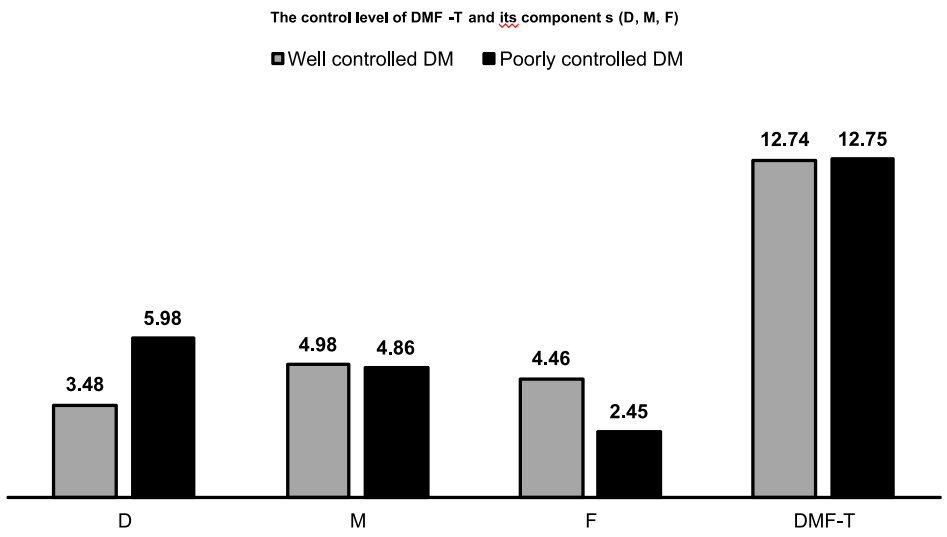

Fig. 5. The control level of DMF-T and its components (D, M, F)

This study of adolescents with type 1 DM demonstrated that none of the subjects has intact dentitions. Poor glicaemic control and the early onset of DM may increase the risk of dental caries but appropriate oral hygiene together with satisfactory metabolic control may prevent the development of dental caries in adolescents with type 1 DM.

4. Tooth eruption

Eruption is the phisiological process of tooth development and growth during which the teeth enter the mouth and become visible. Normally they cut themselves throught the oral mucosa without any inflammation.

Mixed dentition starts when the first permanent molar appears in the mouth, usually at five or six years, and ends when the last primamry tooth is lost, usually at ten, eleven, or twelve years.[92] Afterwards the permanent dentition begins and lasts as long as the person lives or until all of the teeth are lost (edentulism).

4.1 Diabetes mellitus and accelerated eruption

It has been demonstrated that diseases containing metabolic instabilities, such as diabetics, weaken the resistance to inflammation[93]. As a result, the gingival inflammation accompanies eruption in the diabetic patients at an increased rate as compared to the non-diabetic individuals.

Information on dentition concerning the age group from 5 to 9 is limited to about 60 type 1 DM children. Ziskin[94] et al. detected a small and insubstantial affect of diabetes on dental development. Studies concerning accelerated dental development in case of diabetic children who are less than 11.5 years old are not in accord. Older diabetic children manifested delayed dental development.[95] The edentulous interval was longer in case of diabetic children than in case of the control population.

Adler et al.[96] proposed that metabolic disorders cause the acceleration and delay experienced in dentition. A biphasic effect of the diabetic state on dental development was detected: on the one hand acceleration in the early diabetes, and on the other retardation in lasting diabetes.[92] The result conflicted with common knowledge: acceleration was observed in dentition until the age of 10 and delay after the age of 10 (especially for the eruption of canine and the premolars).[26] This may be in correlation with diseases containing metabolical instabilities, such as diabetes.

Other studies showed that children with diabetes in the late mixed dentition period (ages 10-14) had a higher inclination for advanced tooth eruption than those without diabetes. Alterations in tooth eruption were not detected in the early mixed dentition group (ages 6-10). Individuals with higher height showed higher propensity for expedited tooth eruption. The expetited eruption among patients with higher height was more characteristic in the older group than in the younger one.[97]

5. Diabetes and dental, periodontal prevention in adolescents

Diabetes is a chronic metabolic disease which affects the entire organism, disturbing especially the oral health. Health habits are substantial for preventing dental and periodontal diseases and maintaining oral health in a population of patients with type 1 DM.

It is a vital task for dentists to foster good oral health habits, executing periodic dental examinations and ensuring sufficient oral hygiene. These conditions significantly affect the diabetic patients' oral health. Dentists must minimize the risk factors of periodontal disease, caries and oral soft tissue pathologies.[88] Dentists should continually instruct and motivate the patient concerning oral hygiene.

Dental practice showed that health promoting methods reduce the patient's smoking habits.[98] It is possible to use pharmacological and behavioral strategies in a private practitioner's office to help patients quit smoking.[99] However inadequate undergraduate dental education and practitioner continuing education threaten to be an obstacle for the daily use of these practices.[100] There are positive signs conserning the improvement of educational opportunities, nevertheless the incorporation of these technics in the clinical practice must still be highlighted.[101]

There are two kinds of professional oral hygiene promotion: active and sustaining procedure. The initial or causal promotion is to eliminate the plaque and the plaque retentional factors and to remove the supra- and subgingival calculus, as well as the root planing and the curettage and to eliminate the carious lesions. The goal of these procedures is to terminate the inflamation of the gingiva and to stop further development of the gingival recession, thus eliminating the bacterial elements responsible for inflammation. The most important condition of efficient private oral hygiene is the smooth cleanable tooth surface. For it is useless to instruct the patient even on the most refined tooth brushing technique or on the use of dental floss if the teeth are covered with calculus or there are overfilled interdental spaces. Dental practice should include dental check within one or two months, oral hygiene treatment, repeated fixing of gingival and periodontal indexes, as well as their comparison with the initial values, and in certain cases causal surgical periodontal interventions. Nontheless, further instructions and motivations are also essential.

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Impact of Hyperglycemia on Xerostomia and Salivary Composition and Flow Rate of Adolescents with Type 1 Diabetes Mellitus

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1. Introduction

Xerostomia is defined as a subjective sensation of having a dry mouth (Fox et al., 1987) and is reported by the patient (Guggenheimer & Moore, 2003; Moore et al., 2001). The subjective feeling of dry mouth (xerostomia) is one of the oral manifestations of diabetes (Sreebny et al., 2006; von Bültzingslöwen et al., 2007). Xerostomia results from a reduction in saliva secretion, although it may occur in spite of the presence of a normal salivary flow rate (Guggenheimer & Moore, 2003; Scully, 2003). Altered saliva composition rather than the quantity of saliva may play a role in the induction of xerostomia (Anttila et al., 1998; Fox, 1996).

Type 1 diabetes mellitus (DM1) is a metabolic dysfunction characterized by hyperglycemia resulting from definitive deficiency in insulin secretion caused by autoimmune illness and genetic factors (ADA, 2004). The American Diabetes Association (ADA) reports that 75% of DM1 cases are diagnosed in persons under the age of 18 years (ADA, 2006). Glycemic control is fundamental to the management of diabetes and is associated with sustained decreased rates of microvascular (retinopathy and nephropathy) as well as neuropathic complications (ADA, 2008). Glycemic control has a modifying effect on the relation between dental caries and salivary factors in young patients (Syjälä et al., 2003).

Patients with DM1, particularly those who have poor glycemic control, may have decreased salivary flow rate (Guggenheimer & Moore, 2003). Many clinical problems develop in the presence of xerostomia, such as: difficulty in swallowing and speech, high susceptibility to oral infections (mainly candidiasis and dental caries), gingivitis and mucositis (Anttila et al., 1998). Furthermore, xerostomia was shown to have a negative impact on the quality of life of adolescents with DM1 (Busato et al., 2009).

The relationship among DM1, salivary composition and xerostomia has been widely investigated (Swanljung et al., 1992; Moore et al., 2001; López et al., 2003; Siudikiene et al., 2006; Siudikiene et al., 2008; Orbak et al., 2008). It has been found that most DM1 patients have salivary dysfunction as well as differences in biochemical salivary composition compared with healthy subjects (Swanljung et al., 1992; Moore et al., 2001; López et al., 2003; Siudikiene et al., 2006; Siudikiene et al., 2008; Orbak et al., 2008). Moreover, there is a lack of studies showing the relationship among hyperglycemia, xerostomia and salivary factors, especially in

adolescents with DM1. Thus, the aim of this study was to evaluate the association among hyperglycemia, xerostomia, salivary flow and composition of adolescents with DM1.

1.1 Materials and methods

This study was approved by the Research Ethics Committee of the Pontifical Catholic University of Paraná and by the Management of the Paraná Federal University Teaching Hospital. Patients and their parents or guardians were informed about the objective and the other aspects of the study and signed a Term of Independent Informed Consent.

1.1.1 Study groups and study design

A case-control epidemiologic study was performed on adolescents, allocated between two groups: control group, comprised of 51 non-diabetic subjects who were recruited from public high schools, and DM1 group, comprised of 51 adolescents with DM1, who receive follow-up at the Diabetes Outpatients Department of the Paraná Federal University Teaching Hospital. DM1 group and control group were paired regarding gender and age (14 – 19 years). DM1 diagnosis using the ADA (ADA, 2004) classification was established as a criterion for inclusion in DM1 group. The criterion for inclusion in control group was that of non-diabetic adolescents who had not used any medication for at least one month. The exclusion criteria used for both groups were: presence of systemic conditions that could influence salivary gland physiology, psychotropic drugs users, smokers, illicit drugs users or alcohol users (Busato et al., 2009).

1.1.2 Glycemic control

The results of postprandial capillary glucose (CG) tests performed at the time of saliva collection were recorded. Patients with good glycemic control were considered to be those with CG values of ≤ 130 mg/dL (DM1-A group), whereas hyperglycemic patients were considered to be those with CG values of > 130 mg/dL (DM1-B group) (ADA, 2006; 2008).

1.1.3 Xerostomia

Xerostomia was defined as a dry mouth sensation, reported by the subject. The subjects were asked if they had had a dry mouth sensation in the last six months (question A). If the answer was positive to xerostomia, they were also asked if it had occurred constantly during the last six months. Xerostomia was considered to exist if it had occurred daily during the six-month period. This evaluation was completed by the following questions: How would you describe the amount of saliva in your mouth? (question B). Do you have difficulty in swallowing food? (question C). Do you need to have something to drink in order to be able to swallow your food? (question D) (Carda et al., 2006).

Xerostomia was weighted according to three scales of perception: xerostomia 1 (dry mouth), when the answer to question A was "yes"; xerostomia 2, when there was a positive answer for question A and one other question (B, C or D); xerostomia 3, when there was a positive answer to question A and to two or more questions relating to xerostomia (B, C or D).

1.1.4 Saliva collection and treatment

Salivary flow was evaluated by means of stimulated saliva collection. The method used was that of mechanical masticatory stimulation, using a piece of sterile rubber tourniquet of a standardized size (1.5 cm), masticated continuously by the patient for six minutes. Saliva

produced during the first minute of stimulation was discarded. During the following five minutes, the patient expelled saliva into a sterilized universal collecting recipient that had been previously weighed using Marte® analytical scales, model AL 500 (São Paulo-SP/Brazil). The saliva was collected between 8 a.m. and 10 a.m. Stimulated saliva flow rate (SSFR) was evaluated by means of the gravimetric method and expressed in mL/min (Banderas-Tarabay et al., 1997).

The remaining saliva samples were centrifuged (3,000 g for 10 min). Total protein and calcium salivary concentrations were determined using a colorimetric method (LABTEST® kits/Vista Alegre-MG/Brazil). Amylase salivary concentrations were determined by a kinetic colorimetric method (LABTEST® kits/Vista Alegre-MG/Brazil). Urea salivary concentrations were determined by an enzymic colorimetric method (LABTEST® kits/Vista Alegre-MG/Brazil). Salivary glucose was analysed by an enzymic colorimetric method (BIOCLIN® kits/Belo Horizonte-MG/Brazil). The determination of the salivary concentrations was performed three times.

1.1.5 Statistical analysis

The data were analysed using SPSS version 15.0 for Windows. Normality analysis was performed using the Kolmogorov-Smirnov Test, and the Levene test was used to analyse variance homogeneity. The other tests used were Mann-Whitney test and Fisher's exact test considering statistically significant values ($p \leq 0.05$ and CI 95%).

2. Results and discussion

A total of 102 subjects were included in this study: 51 patients with DM1 (DM1 group) and 51 subjects without DM1 (control group). Twenty-seven subjects were female (52.9%) and 24 were male (47.1%) in each group (DM1 group and control group). Average age was 17 years (14-19, SD = 1.4) in both groups. In DM1 group, average CG was 200.5 mg/dL (SD = 108.09).

In the present study, hyperglycemia (CG > 130 mg/dL) was observed in 33 (65%) adolescents with DM1 (DM1-B group) while 18 (35%) showed good glycemic control (DM1-A group). DM1, regardless of glycemic control, was a risk factor for higher xerostomia prevalence and increased glucose salivary concentrations. Hyperglycemia was a risk factor for SSFR reduction and increased urea and calcium salivary concentrations.

The presence of xerostomia 1 (dry mouth) was indicated by 27/51 (53%) subjects in DM1 group, and 8/51 (16%) subjects in control group ($P < 0.001$) (Table 1). A total of 12 subjects (24%) stated the need to drink liquids during meals in DM1 group in contrast to 2 (4%) subjects in control group ($P = 0.004$). There were no significant differences between DM1 group and control group for the following questions: difficulty in swallowing food and amount of saliva perceived ($P > 0.05$). Only DM1 group subjects presented xerostomia 2 (n=10, 20%) and xerostomia 3 (n=5, 10%). There were significant differences between DM1 group and control group regarding xerostomia 2 ($P = 0.001$) and xerostomia 3 ($P = 0.028$) (Table 1).

Among well-controlled adolescents (DM1-A group), 11/18 (61%) subjects reported xerostomia 1, in contrast to 16/33 (48%) hyperglycemic adolescents (DM1-B group). There were significant differences between DM1-A and control group, and DM1-B and controls for

xerostomia 1 ($P < 0.05$) (Table 2). Table 2 shows the mean and the standard deviations of the salivary concentrations of total protein, amylase, urea, calcium and glucose in DM1, DM1-A, DM1-B and control groups. There were significant differences between DM1 and control groups for salivary concentrations of total protein ($P = 0.009$), calcium ($P = 0.001$), and glucose ($P = 0.021$). There were significant differences regarding total proteins ($P = 0.007$) and glucose ($P = 0.024$) salivary concentrations when DM1-A group was compared with control group. DM1-B group (adolescents with hyperglycemia) showed higher urea ($P = 0.042$), calcium ($P < 0.001$), and glucose ($P = 0.038$) salivary concentrations compared with controls.

Variables n (%)		DM1-group n = 51	Control group n = 51	*P* value
Xerostomia 1 (dry mouth)	Yes	27 (53)	8 (16)	<0.001 †
	No	24 (47)	43 (84)	
Need to drink	Yes	12 (24)	2 (4)	0.004 †
	No	39 (76)	49 (96)	
Amount of saliva perceived	Low	5 (10)	2 (4)	NS
	Normal	46 (90)	49 (96)	
Difficulty in swallowing	Yes	3 (6)	1 (2)	NS
	No	49 (94)	50 (98)	
Xerostomia 2	Yes	10 (20)	0 (0)	0.001 †
	No	41 (80)	51 (100)	
Xerostomia 3	Yes	5 (10)	0 (0)	0.028 †
	No	46 (90)	51 (100)	

DM1-group: adolescents with DM1, Control group: adolescents without DM1

† Fisher's exact test $P \leq 0.05$, NS non-significant ($P > 0.05$)

Table 1. Characteristics of xerostomia of the studied population

Variables N (%) or mean (SD)	DM1-group n = 51	DM1-A n=18	DM1-B n=33	Control group n = 51	*P* value
Xerostomia 1					
Yes	27 (53)	11 (61)	16 (48)	8 (16)	<0.001[a c] †
No	24 (47)	7 (39)	17 (52)	43 (84)	0.001[b] †
SSFR (mL/min)	0.932 (0.537)	1.140 (0.688)	0.812 (0.361)	1.224 (0.577)	0.003[a] Ŧ NS[b] 0.002[c] Ŧ
Total Protein (mg/dL)	218 (386)	139 (67)	262 (460)	239 (144)	0.009[a] Ŧ 0.007[b] Ŧ NS[c]
Amylase (U/dL)	758 (33)	767 (35)	757 (31)	778 (9)	NS[a b c]
Urea (mmol/L)	5.340 (2.157)	4.662 (1.565)	5.769 (2.140)	4.957 (2.040)	NS[a b] 0.042[c] Ŧ
Calcium (mmol/L)	0.752 (0.496)	0.562 (0.366)	0.803 (0.515)	0.401(0.338)	0.001[a] Ŧ NS[b] <0.001[c] Ŧ
Glucose (mmol/L)	0.174 (0.183)	0.158 (0.154)	0.170 (0.189)	0.098 (0.115)	0.021[a] Ŧ 0.024[b] Ŧ 0.038[c] Ŧ

DM1-group: adolescents with DM1; DM1-A: adolescents with DM1 (CG ≤ 130mg/dL); DM1-B: adolescents with DM1 (CG > 130mg/dL); and Control group: adolescents without DM1
SSFR (stimulated salivary flow rate)
Ŧ Mann-Whitney U test, † Fisher's exact test, NS non-significant (P > 0.05)
[a] p value of DM1-group X control group; [b] p value of DM1-A X control group; and [c] p value of DM1-B X control group

Table 2. Salivary characteristics and xerostomia of the studied population

In the present study, xerostomia 1 prevalence was demonstrated in 27 (53%) adolescents with DM1 (DM1 group): 11 (61%) with good glycemic control and 16 (48%) with hyperglycemia, in contrast to 8 (16%) non-diabetes ones (control group) (Tables 1 and 2). Xerostomia was significantly associated with DM1 (Table 1) regardless of hyperglycemia (Table 2). Xerostomia 2 and xerostomia 3 only occurred in DM1 group, demonstrating that xerostomia is one of the oral manifestations of diabetes (Sreebny et a.l, 2006; von Bültzingslöwen et al., 2007). Xerostomia prevalence in elderly diabetic patients varies from

24.1% in patients with DM1 (Moore *et al* 2001) up to 76.4% in patients with type 2 DM (Carda et al., 2006). Moreover, there are limited accounts in the literature regarding the prevalence of xerostomia in adolescents with DM1, which makes direct comparisons between our study and other studies in adults difficult.

The need to drink liquids during meals was reported by 12 (24%) adolescents with DM1 and by 2 (4%) adolescents without diabetes (P = 0.004, Table 1). Nevertheless, in spite of this relationship between DM1 and "need to drink", it should be emphasized that family and individual habits may be related to this relationship. The habit of drinking juices, soft drinks or even water during meals is very common and frequently does not indicate a real necessity to drink in order to be able to swallow food. The clinical importance of the need to drink during meals among adolescents with DM1 needs to be further investigated in other studies.

In this study, average SSFR was 0.932 mL/min in DM1 group and 1.224 mL/min in control group (P = 0.003). In DM1-A group, average SSFR was 1.140 mL/min, presenting no significant difference compared with controls (P > 0.05) (Table 2). In the hyperglycemic subjects group (DM1-B), average SSFR was 0.812 mL/min. There was significant difference for SSFR between DM1-B and control groups (P = 0.002) (Table 2). Average SSFR values vary from 0.79 mL/min in children and adolescents with DM1 (Belazi et al., 1998) reaching 1.17 mL/min in adolescents with DM1 (Siudikiene et al., 2006). The latter value (Siudikiene et al., 2006) is similar to the average SSFR in well-controlled adolescents in the present study (DM1-A group, 1.140 mL/min, Table 2). The average SSFR value was significantly different between DM1 group (0.932 mL/min) and control group (1.224 mL/min), which is in consonance with previous studies with adolescents with DM1 (Siudikiene et al., 2006, 2008). In the present study, hyperglycemia was associated to a reduction in salivary flow (Table 2). This result agrees with a previous study, where it was suggested that it might be that the overall dehydration associated with hyperglycemia decreased the volume of saliva excreted (Karjalainen et al., 1996). Low salivary flow can influence increased caries experience in DM patients (Siudikiene et al., 2006, Márton et al., 2008). Furthermore, the subjective feeling of dry mouth (xerostomia) may result from a reduction in saliva secretion and was shown to have a negative impact on the quality of life of adolescents with DM1 (Busato et al., 2009).

Saliva contains immunological and non-immunological proteins with antibacterial properties (Humphrey & Williamson, 2001). In this study, good glycemic control (DM1-A group) was associated to a decrease in total proteins salivary concentration compared with controls. Conversely, there was no significant difference for salivary concentration of total proteins in the presence of hyperglycemia (DM1-B group) compared with non-diabetic subjects (control group). Moreover, amylase salivary concentration in adolescents with DM1 did not show significant differences compared with controls. Previous studies (Twetman et al., 2002; Mata et al., 2004; Carda et al., 2006; Moreira et al., 2009) have shown significant differences in total proteins salivary concentrations between subjects with and without DM1. Others studies are needed to further investigate the association of hypoglycemia with total proteins salivary concentrations in adolescents with DM1.

Salivary calcium concentration has a fundamental role in maintaining tooth integrity though the modulation of remineralization and demineralization (Humphrey & Williamson, 2001). In the present study, hyperglycemia was associated to an increase in salivary concentration

of calcium (Table 2), and calcium salivary concentration in DM1 group was significantly higher compared with that of control group, in consonance with a previous study (Mata et al., 2004).

3. Conclusion

In this study, the glucose salivary concentration was significantly higher in DM1, DM1-A and DM1-B groups when each one was compared with control group. Some studies (Belazi et al., 1998; López et al., 2003; Moreira et al., 2009) have shown this difference, whereas others studies (Swanljung et al., 1992; Carda et al., 2006) have not found difference in glucose salivary concentrations between subjects with and without DM1. The increased concentrations of glucose in the saliva of adolescents with DM1 may be important for controlling and monitoring the disease. It may possibly be related to blood glucose (Belazi et al., 1998; Iughetti et al., 1999; Mata et al., 2004).

There were no significant differences for urea salivary concentrations between adolescents with DM1 (DM1 group) and without DM1 (control group), which is in accordance with a previous study (Meurman et al., 1998) and contradicts others (López et al., 2003; Carda et al., 2006). Moreover, in the latters (López et al., 2003; Carda et al., 2006), subjects with DM1 showed higher urea salivary concentrations compared with controls.

In the present study, hyperglycemia was associated with an increased urea salivary concentration, with significant difference between DM1-B and control groups (Table 2). Hyperproteic diet and dysfunction of urea excretion due to renal failure may increase urea values in plasma and urine (Searcy et al., 1964). Future studies are needed to further investigate the relationship among the increased values of urea salivary concentration in adolescents with hyperglycemia, renal dysfunction and diet.

The significant difference in salivary composition and SSFR between adolescents with and without DM1 and the significantly higher xerostomia prevalence noted in adolescents who have DM1 may suggest an increased risk of dental caries and oral disease in DM1 patients. Furthermore, xerostomia has been shown to have a negative impact on the quality of life of adolescents with DM1 (Busato et al., 2009).

Moreover, there are limited accounts in the literature regarding the prevalence of hyperglycemia and its association with salivary composition, flow rate and xerostomia in adolescents with DM1, which makes direct comparisons between our study and other studies difficult.

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21

The Effect of Type 1 Diabetes Mellitus on the Craniofacial Complex

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1. Introduction

Diabetes mellitus (DM) is one of the systemic diseases affecting a considerable number of patients (Bensch *et al.*, 2003). Numerous experimental and clinical studies on the complications of diabetes mellitus have demonstrated extensive alterations in bone and mineral metabolism, linear growth, and body composition (Giglio and Lama, 2001). DM is a metabolic disorder characterized by disturbed glucose metabolism, manifesting primarily as chronic hyperglycemia.

Bone metabolism is impaired in Type 1 Diabetes Mellitus (T1DM), either through the direct effect of insulin deficiency or hyperglycaemia or via the more long-term effects of vascular disease. Previous data suggested that osteoblast function can be corrected by restoration of glycaemic control, indicating that hyperglycaemia produces direct impairment of bone metabolism (Shyng *et al.*, 2001).

Growth is a highly complex mechanism resulting not only in a major change in size, but also in minor changes in shape. The changes in shape of the craniofacial skeleton are of particular interest because pathological growth of certain anatomical regions may lead to functional and esthetic problems. In the craniofacial skeleton, it has been well known that growth involves a mosaic of growth sites that grow at different rates and mature at different times (Vandeberg *et al.*, 2004a; VandeBerg *et al.*, 2004b), its growth and by analogy, the response to growth disruption is much more complex than that of the appendicular skeleton (Vandeberg *et al.*, 2004a). Different parts of the craniofacial complex might be expected to respond differently to the same hormonal or biomechanical stimulus (VandeBerg *et al.*, 2004b). T1DM has been shown to affect the general growth of patients due to insulin deficiency and consequently leads to delayed skeletal maturation (Schwartz, 2003).

2. Types of bone formation in craniofacial complex

Bone forms in two ways, resulting in two types of mature bone – intramembranous and cartilage. Cartilage bone forms in a replacement process within the cartilage models of the

embryo and infant. Intramembranous bone forms through the activation of the osteoblast cell or specialized bone forming cells in one of the layers of fetal connective tissue. The bones of the cranial vault, the face, and the clavicle are intramembranous in origin. All other bones of the body form from cartilage. Intramembranous bone include the mandible, the maxilla, the premaxilla, the frontal bone, the palatine bone, the squamous part of temporal bone, the zygomatic bone, the medial plate of the pterygoid process, the vomer, the tympanic part of the temporal bone, the nasal bone, the lacrimal bone, and the parietal bone. The original pattern of intramembranous bone changes with progressive maturative growth when these bones begin to adapt to environmental influences. This accounts for deformities due to malfunction, disease and other environmental factors (Salzmann, 1979).

3. Causes of growth problems

Growth disturbances can be associated with specific anatomic or functional defects. They may be of endocrine or non endocrine origin and may result from genetic, nutritional or environmental factors. Disturbances in somatic growth show themselves in retardation or acceleration of the skeletal system, including the facial and cranial bones. Causes for growth problems usually fall into the following categories (Kumar, 2009):

- **Familial short stature**
  Familial short stature is a tendency to follow the family's inherited short stature (shortness).
- **Constitutional growth delay with delayed adolescence or delayed maturation**
  A child who tends to be shorter than average and who enters puberty later than average, but is growing at a normal rate. Most of these children tend to eventually grow to approximately the same height as their parents.
- **Illnesses that affect the whole body (Also called systemic diseases)**
  Constant malnutrition, digestive tract diseases, kidney disease, heart disease, lung disease, hepatic disease, diabetes, and severe stress can cause growth problems.
- **Endocrine (hormone) diseases**
  Adequate production of the thyroid hormone is necessary for normal bone growth. Cushing's syndrome can be caused by a myriad of abnormalities that are the result of hypersecretion of corticosteroids by the adrenal gland. Growth hormone deficiency involves a problem with the pituitary gland (small gland at the base of the brain) that secretes several hormones, including growth hormone.
- **Congenital (present at birth) problems in the tissues where growth occurs**
  A condition called intrauterine growth restriction (IUGR), slow growth within the uterus occurs during a pregnancy. The baby is born smaller in weight and length than normal, in proportion to his/her short stature.

4. Outline of studying the effect of diabetes mellitus on craniofacial growth

Approximately 60% of adult bone mass including craniofacial bone is gained during the peak of the growth period which coincides with the onset of T1DM condition affecting the bone formation process (Eastell and Lambert, 2002). It is worth mentioning here that although T1DM condition exact etiological factors are totally unknown however; understanding the course of T1DM condition and its impact on craniofacial development may lead to improving the oral health for a large sector of the population worldwide.

Numerous experimental and clinical studies on the complications of DM have demonstrated extensive alterations in bone and mineral metabolism, linear growth, and body composition. Investigators in the fields of bone biology including orthodontics have long been interested in the general causes that affect the normal growth of the craniofacial region. T1DM has been shown to affect the general growth of patients with earlier onset of the disease, especially onset before or around the circumpubertal growth spurts (Chew, 1991).
In general, growth of the craniofacial complex is controlled by genetic and environmental factors (Giglio and Lama, 2001; Yonemitsu *et al.*, 2007). Regulatory mechanisms responsible for normal morphogenesis of the face and head involve hormones, nutrients, mechanical forces, and various local growth factors. The poor growth and alterations in bone metabolism have been associated with T1DM in both humans and experimental animals (Giglio and Lama, 2001). It is of prime importance investigating the changes in craniofacial bone structure and dynamic bone formation in DM condition to explore the impact of the diabetic condition on various mandibular growth elements and bone quality.

The following parts of this chapter are going to focus on these points:

- Investigating the effects of juvenile diabetes on general craniofacial growth and skeletal maturation.
- Analyzing the pattern of association between craniofacial morphology and skeletal maturation.
- Determination of the changes in bone morphology in diabetic rat mandible using micro-C.T.
- Determination of the mineral apposition rate and the bone formation rate in diabetic rat mandible using histomorphometric analysis.

5. Animal and experimental diabetic model

The animal studies using diabetic model presents various advantages when compared to studies carried out on human diabetic cases. Human studies can be limited by small sample sizes, cross-sectional designs, uncontrolled variables, and often retrospective nature, animal models have been used to yield more rigorous analyses (Singleton *et al.*, 2006).

5.1 Diabetic model

Experimental diabetic models include the streptozotocin-induced diabetic rat and the spontaneously diabetic BioBreeding rat. The occurrence of different abnormalities indicating altered bone formation after inducing DM with streptozotocin (STZ) is well documented (Giglio and Lama, 2001; Hough *et al.*, 1981; Tein *et al.*, 1998). Streptozotocin-induced diabetes mellitus (STZ-DM), caused by the destruction of pancreatic β-cells and is similar to T1DM in human, is characterized by mild to moderate hyperglycemia, glucosuria, polyphagia, hypoinsulinemia, hyperlipidemia, and weight loss. STZ-DM also exhibits many of the complications observed in human DM including enhanced susceptibility to infection and cardiovascular disease, retinopathy, alterations in angiogenesis, delayed wound healing, diminished growth factor expression, and reduced bone formation (Lu *et al.*, 2003).

5.2 Importance of testing the uncontrolled diabetic condition

In the usual clinical situation, although T1DM patient is treated with insulin, patient may still suffer from an overall poor diabetic metabolic state with an uncontrollable blood glucose level and a high and sometimes changing insulin requirement (Follak *et al.*, 2004).

5.3 Inducing diabetic condition

All the experimental protocols followed had been approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University, and the experiments were carried out under the control of the University's Guidelines for Animal Experimentation. In our investigation we explored the various effects of DM using the streptozotocin DM model. Twelve 3-week old male Wistar rats were used for this study. They were randomly divided into two groups, the control group and the diabetes group (DM group), each group consists of 6 rats. The rats in the control group were injected intra-peritoneal with a single dose of 0.1M sodium citrate buffer (pH 4.5), while the rats in the DM group were injected intra-peritoneal with a single dose of citrate buffer containing 60mg/kg body weight of streptozotocin (Sigma Chemical Co., St. Louis, MO, USA).(Alkan *et al.*, 2002; McCracken *et al.*, 2006; Shyng *et al.*, 2001; Tein *et al.*, 1998) All animals were fed on standard Rodent diet (Rodent Diet CE-2; Japan Clea Inc., Shizuoka, Japan) with free access to water. Body weights, the presence of glucose in urine and blood glucose levels were recorded on day 0, 2, 7, 14, 21 and 28 after STZ injection.

Diabetes condition was determined by the presence of glucose in urine and blood. The urine of the rats was tested using reagent strips (Uriace Ga; TERUMO). (Abdus Salam *et al.*, 2004; Matin *et al.*, 2002) Blood samples of the rats were obtained via vein puncture of a tail vein, and blood glucose levels were determined using a glucometer (Ascensia Brio. Bayer Medical). Positive urine test and a blood glucose level greater than 200 mg/dl was considered DM.(Giglio and Lama, 2001).

Fig. 1. shows the weights of the rats (mean±SD) in both groups. DM group showed a significant decrease in weight. After STZ injection by 48 hours the urine test showed that the entire DM group had a high glucose level and this was confirmed by the high blood glucose measurements as shown in Fig. 2. A Student's t-test was used to compare the mean of weights and blood glucose levels in both groups.

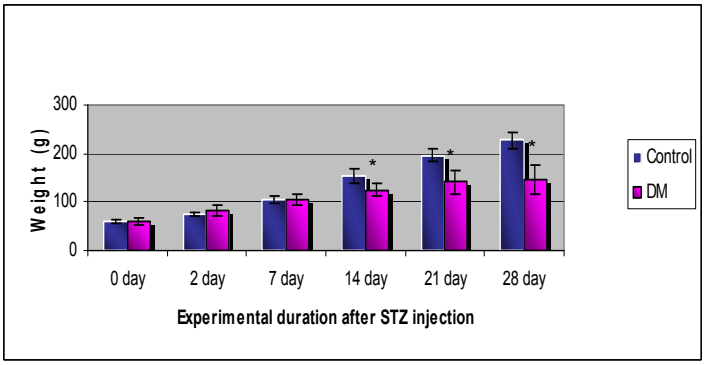


Fig. 1. Comparison between the changes of the rat's weight in the control and DM group. The weights of the DM group are significantly decreased as compared to the weights of the control group (*:$p<0.05$).

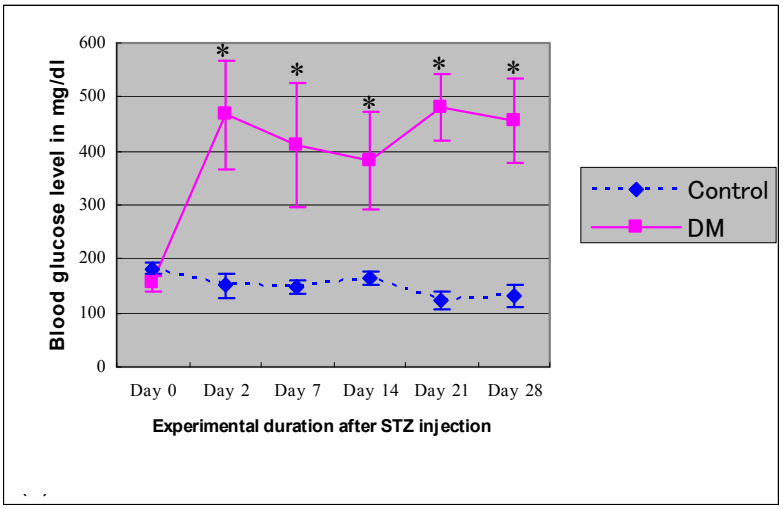


Fig. 2. Line graph represents the blood glucose levels for the control and DM group. The blood glucose level in DM group increased significantly 48h post-STZ injection and during the entire experimental period. Values are mean±SD. Significant differences between the two groups are marked with asterisks ($p< 0.05$).

6. Analytic studies conducted to test the effect of diabetic condition on craniofacial growth

6.1 Cephalometric analysis

Cephalometric measurements are still one of the most widely spread diagnostic aids crucial for the diagnosis of various abnormalities in the craniofacial complex (Chidiac *et al.*, 2002).

The protocol for examining the cephalometric measurements in DM rats involves the following steps:

- Prior to each radiographic session, the rats are anaesthetized with diethyl ether and intraperitoneal injection of 8% chloral hydrate using 0.5ml/100g of body weight.
- Each animal is then placed in this specially-designed apparatus (Fig. 3) to maintain standardized head posture and contact with the film (SGP-3, Mitsutoy, Tokyo, Japan) where the head of each rat is fixed firmly with a pair of ear rods oriented vertically to the sagittal plane and the incisors are fixed into a plastic ring.
- The settings of lateral and dorsoventral cephalometric radiographs are 50/55kVp, 15/10mA, and 20/60-sec impulses respectively (Singleton *et al.*, 2006; Vandeberg *et al.*, 2004a; VandeBerg *et al.*, 2004b).
- A 10 mm steel calibration rod is incorporated into the clear acrylic table on which the animals are positioned for the radiographs.
- All the radiographs are developed and scanned at high resolution by the same operator (Singleton *et al.*, 2006).

The cephalometric landmarks (Table 1; Fig. 4) were derived from previous studies on rodents (Engstrom *et al.*, 1988; Kiliaridis and Thilander, 1985; Singleton *et al.*, 2006; Vandeberg *et al.*, 2004a; VandeBerg *et al.*, 2004b). Selected linear measurements were then

obtained (Table 2). To ensure reliability and replicability of each measurement, each distance was digitized twice and the two values were averaged.

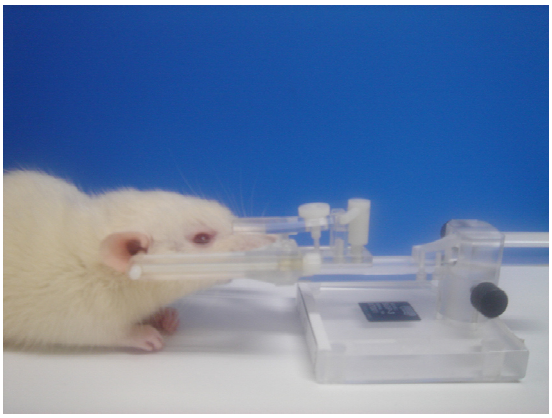


Fig. 3. Apparatus for roentgenographic cephalometry

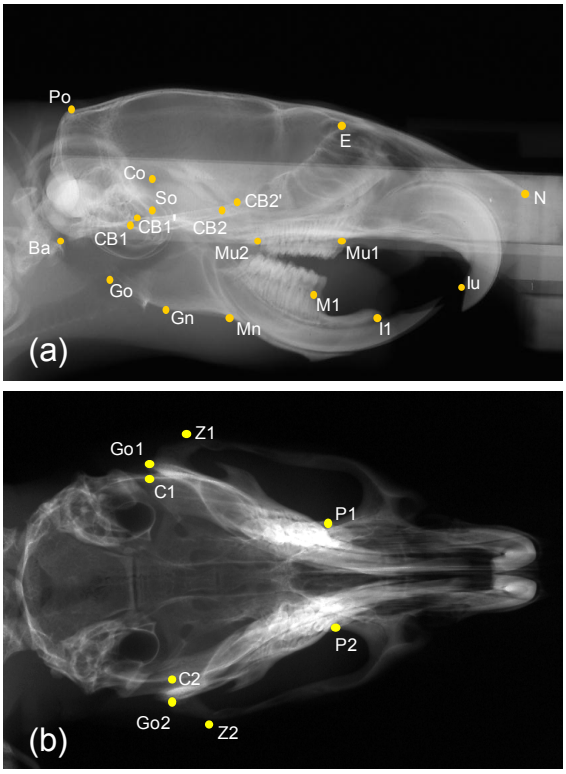


Fig. 4. Location of cephalometric points on radiographs: (A) Sagittal and (B) transverse.

On the sagittal radiograph
N: The most anterior point on the nasal bone
E: The intersection of the frontal bone and floor of anterior cranial fossa
Po: The most posterior and superior point on the skull
Ba: The most posterior and inferior point on the occipital condyle
Co: The most posterior and superior point on the mandibular condyle
Go: The most posterior point on the mandibular ramus
Mn: The most concave portion of the concavity on the inferior border of the mandibular corpus
Gn: The most inferior point on the ramus that lies on a perpendicular bisector of the line Go-Mn
Il: The most anterior and superior point on the alveolar bone of the mandibular incisor
So: The intersection of the most anterior tympanic bulla and the superior border of the sphenoid bone
CB1: The most anterior point on the occipital bone at the spheno-occipital synchondrosis
CB1′ : The most posterior point on the sphenoid bone at the spheno-occipital synchondrosis
CB2: The most anterior point on the sphenoid bone at the spheno-basispheno synchondrosis
CB2′ : The most posterior point on the basisphenoid bone at the spheno-basispheno-synchondrosis
Ml: The junction of the alveolar bone and the mesial surface of the first mandibular molar
Mu1: The junction of the alveolar bone and the mesial surface of the first maxillary molar
Mu2: The junction of the alveolar bone and the distal surface of the third maxillary molar
Iu: The most anterior-inferior point on the maxilla posterior to the maxillary incisors
On the transverse radiograph
Z1 & Z2: The points on the lateral portion of the zygomatic arch that produce the widest width
Go1 & Go2: The points on the angle of the mandible that produce the widest width
P1 & P2: The most anterior and medial points within the temporal fossae that produce the most narrow palatal width
C1 & C2: The points on the cranium that produce the widest cranial width

Table 1. Definition of radiographic points

Neurocranium
Po-N: total skull length
Po-E: cranial vault length
Ba-E: total cranial base length
So-E: anterior cranial base length
Ba-CB1: occipital bone length
CB1′ -CB2: sphenoid bone length
Ba-So: posterior cranial base length
Po-Ba: posterior neurocranium height
Viscerocranium
E-N: nasal length
Mu2-Iu: palate length
CB2-Iu: midface length
E-Mu1: viscerocranial height
Mandible
Go-Mn: posterior corpus length
Ml-Il: anterior corpus length
Co-Il: total mandibular length
Co-Gn: ramus height
Transverse X-ray
Go1-Go2: Bigonial width
C1-C2: Maximum cranial width
P1-P2: Palatal width
Z1-Z2: Bizygomatic width

Table 2. Measurements of craniofacial skeleton

In our studies, evaluation of the craniofacial growth of diabetic rats at the age of 7 weeks was done using lateral and dorsoventral cephalometric radiographs. All of the data in each experiment were confirmed the normal distribution, so a Student's t-test was used to compare the mean of each data recorded in the control group and in the DM group. All statistical analyses were performed at a 5% significance level using statistic software (v. 10; SPSS, Chicago, IL, USA).

6.1.1 Changes in the total skull

The size of total skull, denoted by Po-N, was significantly smaller in the DM group than in the control group.

6.1.2 Changes in the neurocranium

Cranial vault length (Po-E), total cranial base length (Ba-E), anterior cranial base length (So-E), Occipital bone length (Ba-CB1), and posterior cranial base length (Ba-So) showed statistically significant decrease in DM group (Table 3, Figs. 5, 6), while other dimensions exhibited no significant differences.

Po-N	total skull length
Po-E	cranial vault length
Ba-E	total cranial base length
So-E	anterior cranial base length
Ba-CB1	occipital bone length
Ba-So	posterior cranial base length

Table 3. Significant changes in the total skull and neurocranium

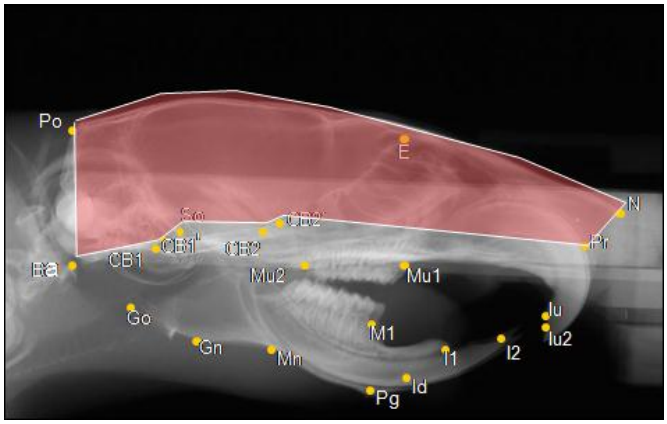


Fig. 5. Neurocranium

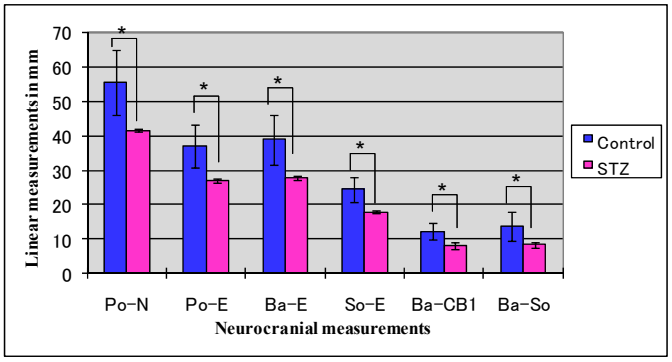


Fig. 6. Changes in the neurocranial measurements of the control and DM group. All the significant measurements are shown in this figure. Values are mean±S.D. Significant differences between the two groups are marked with asterisks (p<0.05).

6.1.3 Changes in the viscerocranium

All measurements of the viscerocranium, including the nasal length (E-N), palatal length (Mu2-Iu), midface length (CB2-Iu), and viscerocranial height (E-Mu1) showed a statistically significant decrease in DM group (Table 4, Figs. 7,8)

E-N	Nasal length
Mu2-Iu	Palate length
Cb2-Iu	midface length
E-Mu1	Posterior viscerocranial height

Table 4. Significant changes in the viscerocranium

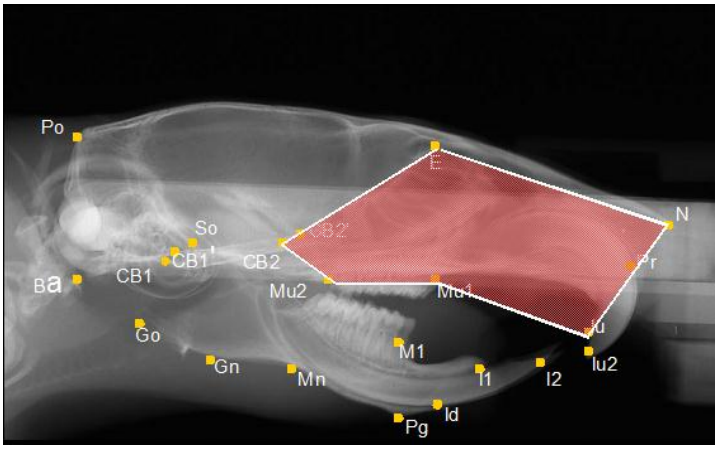


Fig. 7. Viscerocranium

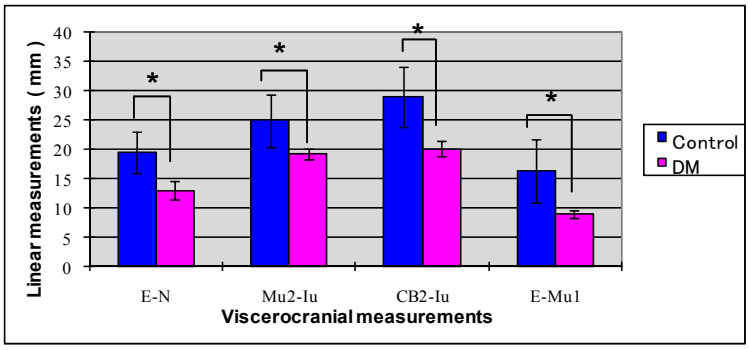


Fig. 8. Changes in the viscerocranial measurements of the control and DM group. All the viscerocranial measurements are significant. Values are mean±S.D. Significant differences between the two groups are marked with asterisks (p<0.05).

6.1.4 Changes in the mandible

In the DM group, the posterior corpus length (Go-Mn), total mandibular length (Co-Il) and the ramus height (Co-Gn) were significantly shorter than in the control group (Table 5, Figs. 9, 10), whereas no remarkable differences were found in the remaining dimensions.

Go-Mn	Posterior corpus length
Co-Il	Total mandibular length
Co-Gn	Ramus height

Table 5. Significant changes in the mandible

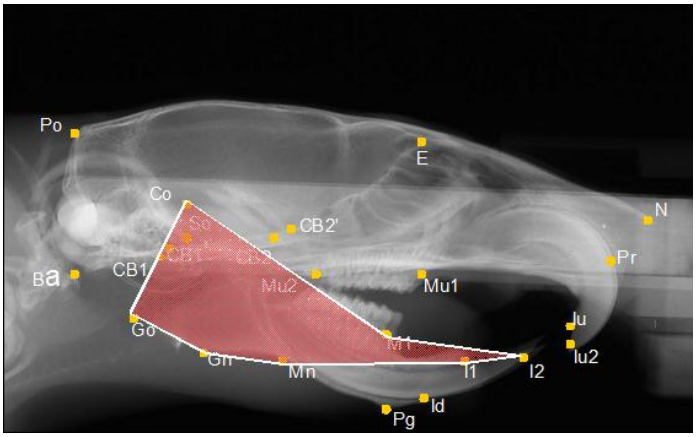


Fig. 9. Mandible

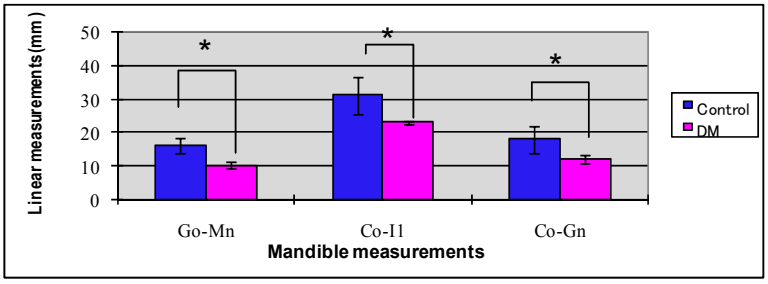


Fig. 10. Changes in the mandible measurements of the control and DM group. Values are mean ± S.D. Significant differences between the two groups are marked with asterisks (p<0.05).

6.1.5 Changes in the transverse X-ray

In transverse X-ray only the maximum cranial width (C1-C2) and the bizygomatic width (Z1-Z2) were statistically decreased in DM group (Table 6, Figs. 11, 12).
All other linear measurements showed no significant differences between both groups

C1-C2	Maximum cranial width
Z1-Z2	Bizygomatic width

Table 6. Significant changes in the transverse X-ray

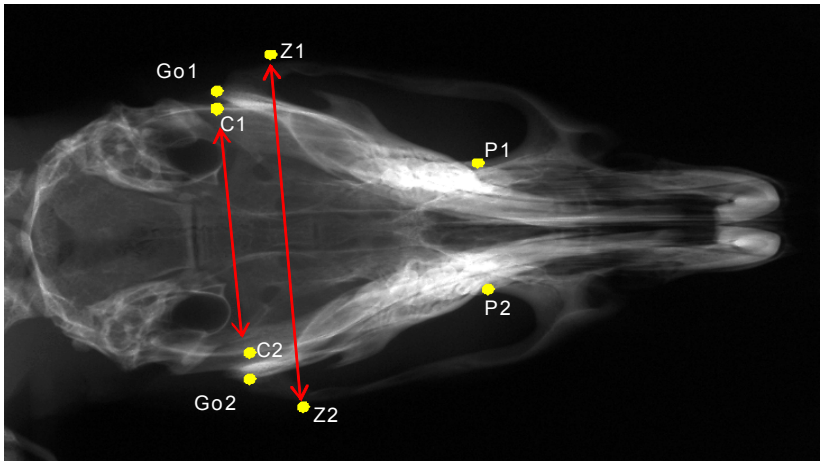


Fig. 11. Transverse X-ray measurements

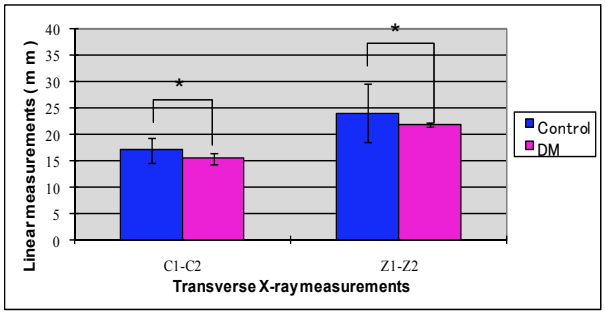


Fig. 12. Changes in the transverse X-ray measurements of the control and DM group. Two measurements in the transverse X-ray were significant. Values are mean±S.D. Significant differences between the two groups are marked with asterisks (p<0.05).

	DM		*C*	
Linear measurements	**Mean**	**SD**	**Mean**	**SD**
Neurocranium				
Po-N	41.67	0.59*	55.61	9.48
Po-E	26.86	0.6*	37.12	6.35
Ba-E	27.74	0.64*	38.87	7.19
So-E	17.91	0.31*	24.41	3.72
Ba-CB1	8.23	1.08*	12.3	2.34
CB1′ -CB2	4.78	0.74	5.93	1.18
Ba-So	8.44	0.7*	13.61	4.23
Po-Ba	10.03	0.83	13.14	2.81
Viscerocranium				
E-N	13.07	1.56*	19.57	3.48
Mu2-Iu	19.34	0.95*	25.01	4.5
CB2-Iu	20.16	1.29*	29	5.07
E-Mu1	8.93	0.69*	16.43	5.36
Mandible				
Go-Mn	10.27	1.04*	16	2.39
M1-I1	5.4	0.88	6.97	1.54
Co-I1	22.95	0.34*	31.13	5.51
Co-Gn	12.22	1.2*	17.9	4.04
Transverse X-ray				
Go1-Go2	18.42	0.42	19.77	1.32
C1-C2	15.43	0.67*	17.03	1.16
P1-P2	8.5	0.41	9.19	0.52
Z1-Z2	21.9	0.33*	24	1.4

*p<0.05 as compared to control values

Table 7. Comparision of skeletal cephalometric measurements for Type 1 Diabetes mellitus (DM) and controls (C)

6.2 Histomorphometric analysis

6.2.1 Fluorescent dyes used for double labeling in histomorphometric analysis

Fluorochromes are calcium binding substances that are preferentially taken up at the site of active mineralization of bone known as the calcification front, thus labeling sites of new bone formation. They are detected using fluorescent microscopy on undecalcified sections. Labeling bones with fluorochrome markers provides a means to study the dynamics of bone formation. The rate and extent of bone deposition and resorption can be determined using double and triple fluorochrome labeling sequences. The sequential use of fluorochromes of clearly contrasting colors permits a more detailed record of events relating to calcification. Flurochromes commonly used in mammals include tetracycline, calcein green, xylenol orange, alirazin red, and hematoporphyrin. Calcein is a fluoresces bright green when combined with calcium (Stuart and Smith, 1992).

6.2.2 Calcein administrations and sections preparation

The steps needed for detecting the double labeling involves the following:

- Rats are subcutaneously injected with 50 mg/kg body weight calcein fluorescent marker on day 21 and day 28 after STZ injection. The time difference between the 2 injections is one week to be able to compare the amount of bone formed during this period (Fig. 14).
- Sacrifice of all animals by transcardiac perfusion under deep anesthesia using 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).
- Mandibles are dissected and fixed in the same solution for 24 hours.
- All specimens are embedded in polystyrene resin (Rigolac, Nisshin EM Co. Ltd., Tokyo, Japan).
- Undemineralized ground frontal sections are processed to show the crown and both apices of buccal and lingual roots of the lower second molar (Shimomoto *et al.*, 2007).

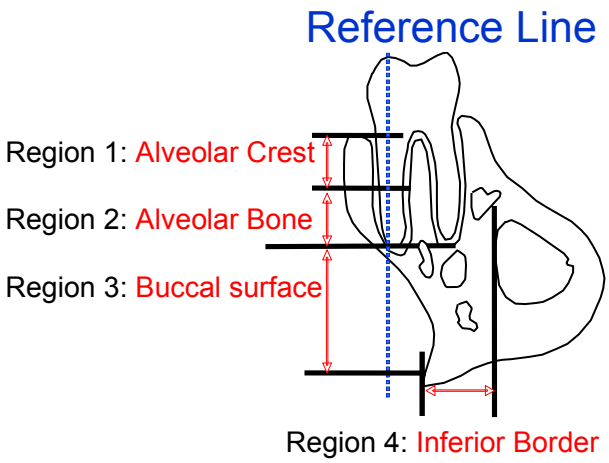


Fig. 13. Schematic drawing of observation regions for dynamic bone histomorphometry. The periosteal surfaces were delimited into 4 areas as alveolar crest (region 1), alveolar bone (region 2), buccal surface of the jaw bone (region 3), and inferior border of the jaw bone (region 4).

6.2.3 Method of analysis

The bone around the lower second molar is centrally located within the mandibular arch, and because of the parallel alignment of the buccal and lingual roots this made a precise reference when frontal sections are produced (Shimomoto *et al.*, 2005). To conduct the histomorphometric analysis it is essential to use a digitizing morphometry system to measure bone formation indices. The system consists of a confocal laser scanning microscope (LSM510, Carl Zeiss Co. Ltd., Jena, Germany), and a morphometry program (LSM Image Browser, Carl Zeiss Co. Ltd., Jena, Germany). Bone formation indices of the periosteal surfaces of the alveolar/jaw bone include mineral apposition rate (μm/day) and bone formation rate ($\mu m^3/\mu m^2$/day), according to the standard nomenclature described by (Parfitt *et al.*, 1987). The calcein-labeled surface (CLS, in mm) is calculated as the sum of the length of double labels (dL) plus one half of the length of single labels (sL) along the entire endosteal or periosteal

bone surfaces; that is, CLS = dL + 0.5sL (Keshawarz and Recker, 1986). The mineral apposition rate (MAR, in μ/day) is determined by dividing the mean of the width of the double labels by the interlabel time (7 days). The bone formation rate (BFR) is calculated by multiplying MAR by CLS (Sheng *et al.*, 1999). Based on the reference line along the long axis of the buccal root, the area superior to the root apex was considered alveolar bone, while the area inferior to the root apex was considered the jaw bone. The lingual side of the bone is excluded, because the existence of the incisor root may influence bone formation.
The periosteal surfaces of the mandible are divided into four regions for analysis (Fig. 13):
Region 1: alveolar crest (upper 1/2 of the tooth root, near the tooth crown)
Region 2: alveolar bone (lower 1/2 of the tooth root, near the root apex)
Region 3: buccal surface of the jaw bone
Region 4: inferior border of the jaw bone

6.2.4 Histomorphometric indices

The obtained results in our study showed that in the alveolar bone (region 2), there was a significant decrease in the MAR (Fig. 15A) BFR (Fig. 15B) recorded in the DM group compared to the control group. However, in the alveolar crest (region 1), the MAR and the BFR in the control and the DM groups were not significantly different. (p<0.05). In the buccal surface (region 3) and inferior borders (region 4) of the jaw bone the MAR (Fig. 15A) and BFR (Fig. 15B) were significantly suppressed compared with those in the control group (p<0.05). Most of the periosteal surfaces in the mandibular regions of the control group showed significantly higher values recorded for the mineral apposition rate and the bone formation rate when compared to the DM group. These results agree with previous studies that recorded diminished lamellar bone formation in DM rats' femur and may suggest an association between the DM condition and the decreased number and function of osteoblasts (Follak *et al.*, 2004; Shyng *et al.*, 2001). The alveolar crest region was the only region that did not show a significant difference in the mineral apposition rate and the bone formation rate parameters among the two groups; this may be attributed to the unique nature of this region exhibiting a highly intensive bone remodeling process especially during the teeth eruption that decreases toward the base of the socket (Gerlach *et al.*, 2002), however further studies are needed to elaborate the detailed pattern of bone growth at the alveolar crest region.

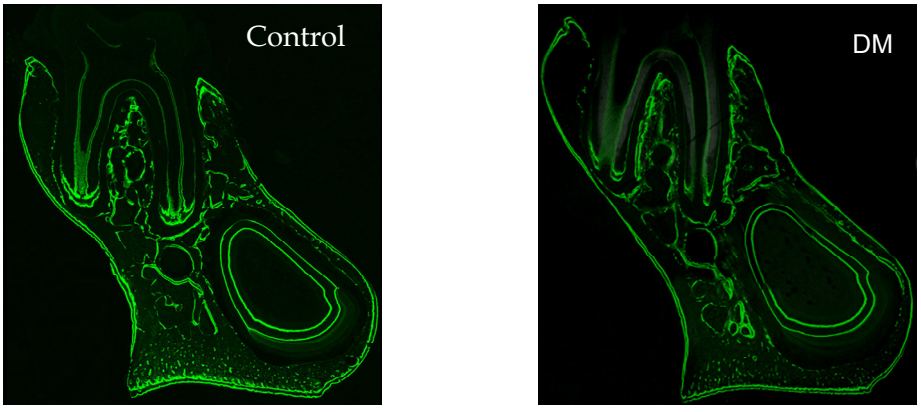
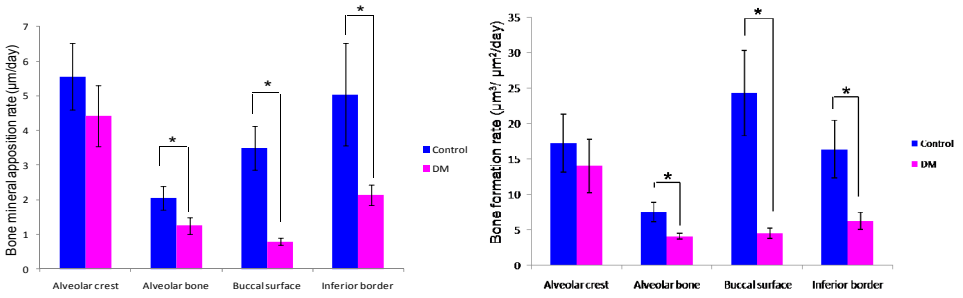


Fig. 14. Frontal sections of the mandibular second molar area. (A) Control; (B) DM. Fluorescent labeling on the periosteal surface indicates new bone formation.



A B

Fig. 15. (A) The changes in mineral apposition rate (MAR) of the mandible between the control group and the DM group. Alveolar crest (region 1, upper 1/2 of the tooth root, near the tooth crown). Alveolar bone (region 2, lower 1/2 of the tooth root, near the root apex). Buccal surface of the jaw bone (region 3). Inferior border of the jaw bone (region 4). The data are expressed as means ± SD. n = 5 for each group. *:p<0.05. (B) The changes in the bone formation rate (BFR/BS) of the mandible between the control group and the DM group. Alveolar crest (region 1, upper 1/2 of the tooth root, near the tooth crown). Alveolar bone (region 2, lower 1/2 of the tooth root, near the root apex). Buccal surface of the jaw bone (region 3). Inferior border of the jaw bone (region 4). The data are expressed as means ± S.D. n = 5 for each group. *:p<0.05.

6.3 Microtomography of the mandible (micro-CT)

Micro-computed tomography (micro-CT) has rapidly become a standard technique for the visualization and quantification of the 3D structure of trabecular bone. Bone architecture and mineralization are generally considered to be important components of bone quality, and determine bone strength in conjunction with bone mineral density.

6.3.1 Protocol adopted to examine the mandible using micro-CT

In our study all specimens were imaged by micro-CT (inspeXio SMX-90CT; Shimadzu Science East Corporation, Tokyo, Japan)

- After removing only the soft tissue, the mandibular plane is set orthogonal to the sample stage.
- Three dimensional images of each hemi mandible are acquired with a resolution voxel size of 15 μm / pixel.
- Raw data are obtained by rotating the sample stage 360 degrees. Then, slice images are prepared using multi-tomographic image reconstruction software (MultiBP; Imagescript, Tokyo, Japan).
- The resulting gray-scale images are segmented using a low-pass filter to remove noise and a fixed threshold to extract the mineralized bone phase.
- The volume of interest is drawn on a slice-based method starting from the first slice containing the crown of the first molar and moving dorsally 100 slices (Laib and Ruegsegger, 1999; Zhang *et al.*, 2008), in the area of the alveolar crest (Between the buccal and lingual roots of the second molar at the cervical region); and the buccal surface of the jaw bone (Shimomoto *et al.*, 2007). Trabecular bone was carefully contoured on the first and the last slice, while the intermediate slices were first interpolated by morphing.

- For observation and analysis of reconstructed 3D images, 3D trabecular structure analysis software (TRI/3D-BON; RATOC System Engineering, Tokyo, Japan) is used (Takada *et al.*, 2006). Reconstructed 3D images were prepared from slice images using the volume rendering method, to analyze the microstructure of the bone (Fig. 16).
- The following parameters are measured: tissue volume (TV), bone volume (BV), bone surface (BS), bone surface / bone volume (BS/BV), bone-volume fraction (BV/TV).
- Four properties of the trabeculae are evaluated: trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), and Trabecular space (Tb.S) (Nakano *et al.*, 2004; Takada *et al.*, 2006).

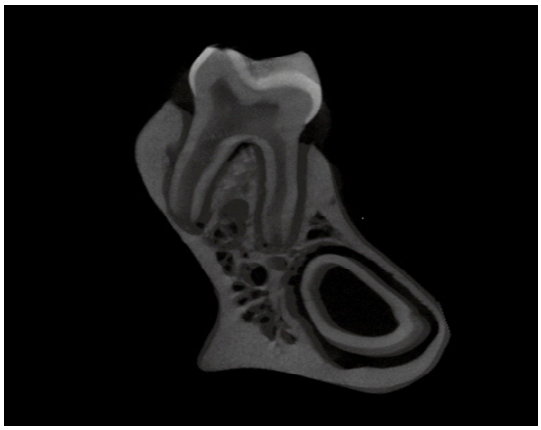


Fig. 16. The left mandible was imaged by micro-CT

6.3.2 Microtomography of the DM mandible

The quantification of micro-CT trabecular bone changes (mean±SD) is shown for the DM and the control groups in (Table 4). All trabecular parameters in both alveolar bone and buccal surface of jaw bone showed significant changes. Compared with the control group, bone volume fraction (BV/TV) was significantly decreased only in the alveolar bone; however, trabecular thickness (Tb.Th) and trabecular numbers (Tb.N) were significantly decreased both in alveolar and buccal surface of jaw bone, in the DM group. Correspondingly, significantly higher trabecular separation (Tb.Sp) and trabecular space (Tb.S) were revealed both in alveolar and buccal surface of jaw bone for the DM group when compared with that of the control group. Also, the bone surface / bone volume (BS/BV) was significantly increased only in alveolar bone (p<0.05). These findings indicate deterioration of the bone quality in the DM group. These results agree with other research work suggesting that the glycaemic levels play an important role in modulating the trabecular architecture especially in mandibular bone (Thrailkill *et al.*, 2005).

The DM condition resulted in alteration of the trabecular distance and thickness as compared to the control group indicating profound impact on the histological integrity of the bone. The reduction in trabecular bone volume accompanied by the expansion of the bone marrow space is in agreement with another investigation (Duarte *et al.*, 2005). In this context, these results may describe a state of osteopenia in experimental diabetic rats, which might be the result of an imbalance between bone formation and resorption

	Alveolar Bone		Buccal Surface Of The Jaw Bone	
	DM	Control	DM	Control
Bone Surface/Bone Volume (1/mm)	36.8±9.5*	63.93±15.3	87.8±5.6	70.0±15.5
Bone Volume/Tissue Volume (%)	22.1±11.6*	46.2±10.2	37.3±4.2	55.2±16.4
Trabecular Thickness (μm)	23.2±2.1*	34.3±4.5	21.5±2.9*	26.1±2.1
Trabecular Number (1/mm)	10.8±1.2*	14.4±2.6	15.2±1.7*	18.9±0.7
Trabecular Separation (μm)	37.5±1.0*	25.5±2.4	38.1±9.1*	25.9±2.4
Trabecular Space (μm)	87.6±4.3*	71.2±11.5	67.7±7.9*	53.1±2.0

*p<0.05 as compared to control values

Table 8. Microarchitectural properties in the mandible as measured by micro-CT

6.4 Histological analysis

6.4.1 Procedure for preparing mandibles for histological analysis

- Mandibles for all groups were decalcified in 10% EDTA solution pH 7.4 for 5 weeks at 4ºC (Yokoyama *et al.*, 2009).
- Specimens are then dehydrated in an ascending ethanol series and embedded in paraffin (Figs. 9, 10, 11).
- Serial horizontal sections (5 μm thick parallel to the occlusal plane) are prepared using a microtome (Leica RM 2155, Nussloch, Germany) (Figs. 12, 13)

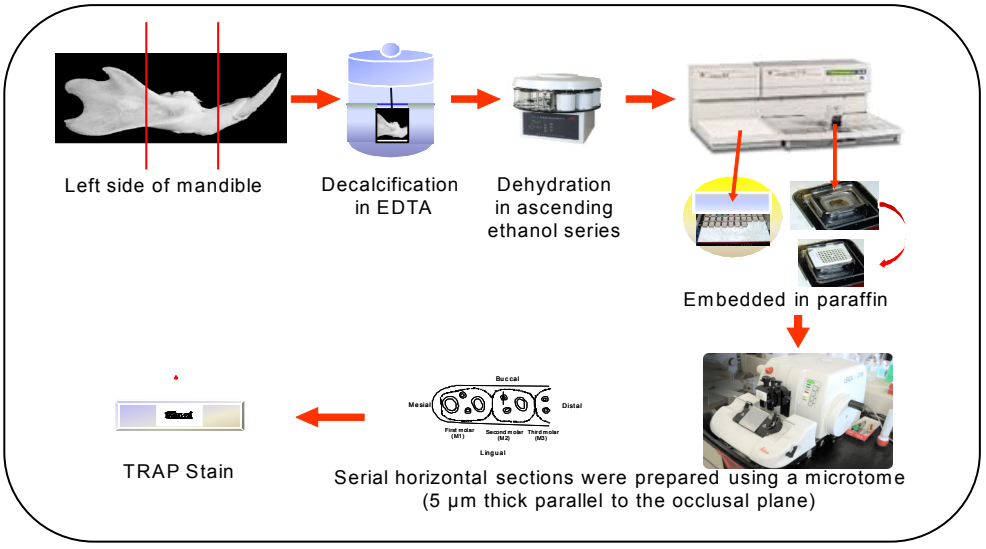


Fig. 17. Experimental procedure for histological section preparation

6.4.2 TRAP staining

Histological sections are incubated for 30–60 min at 37°C in a mixture of 0.8% naphthol AS-BI phosphate (Sigma, St Louis, MO, USA), 0.7% fast red violet salt (Sigma, St Louis, MO, USA) and 50mM sodium tartate diluted in 0.2M sodium acetate buffer (pH 5.4) (Tsuchiya and Kurihara, 1995). Sections were examined under a light microscope. For the histomorphometric assessment of resorption, the number of tartrate-resistant acid phosphatase-positive multinucleated cells (osteoclasts) on the distal surface of the alveolar bone adjacent to the mesio-buccal root of the second molar were counted in each 540 µm x 120 µm area in five consecutive sections, at the middle third of the root selected at least 25µm apart from each specimen (n = 5) of each group (Misawa *et al.*, 2007; Mishima *et al.*, 2002).

6.4.3 Histological analysis

Bone-resorption activity was assessed by counting the number of tartrate-resistant acid phosphatase-positive multinucleate cells (osteoclasts) on the distal surface of the alveolar bone adjacent to the mesio-buccal root of the second molar (Fig. 18A-D). Statistical analysis

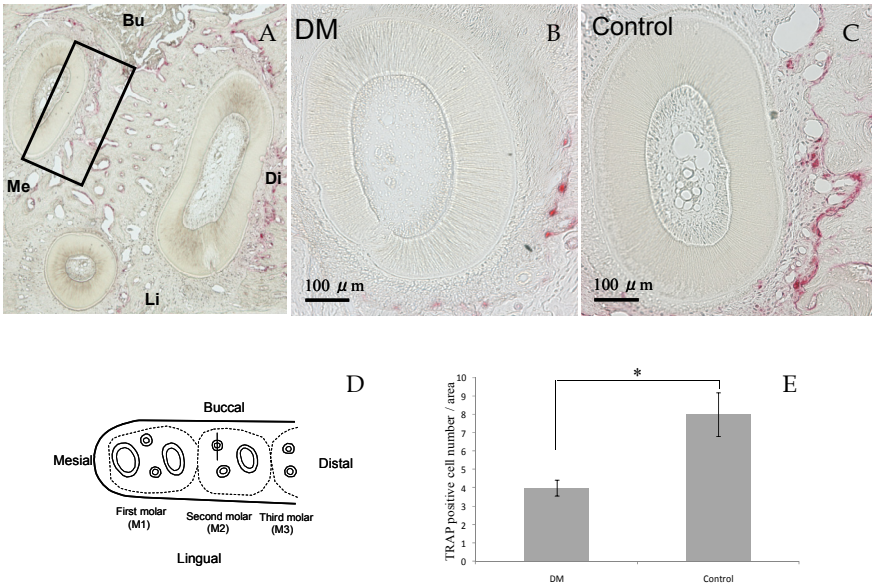


Fig. 18. Osteoclast counts in a horizontal section of the mandibular second molar region stained with Tartarate-resistant acid phosphatase (TRAP). (A) Low magnification photograph of the three roots of the second molar stained with TRAP stain. The black rectangle (540 X 120 µm) indicates the area on the distal surface of the alveolar bone adjacent to the middle third of the mesio-buccal root of the second molar in which the osteoclast cells were counted. Bu, buccal; Li, lingual; Me, mesial; Di, distal. (B) The mesio-buccal root of the control rat (original magnification 100X). (C) The mesio-buccal root of the DM rat (original magnification 100X). (D) A schematic drawing showing the observation area on the distal surface of the alveolar bone adjacent to the mesio-buccal root of the second molar in which the osteoclast cells were counted. (E) The number of TRAP-positive cells on the distal surface of the mesio-buccal root of the mandibular second molar. Values are mean ± SD. *:(p<0.05), bars = 100 µm.

demonstrated a significantly higher number of osteoclast cells in the control group when compared with the DM group (p<0.05) (Fig. 18). Results revealed that the number of osteoclasts was significantly lower in the DM rats than in the controls, in line with previous studies on DM rats' mandible (Mishima *et al.*, 2002) and long bones (Glajchen *et al.*, 1988; Shires *et al.*, 1981). These results confirm that the decreased rate of bone turnover may be associated with the DM condition.

7. Suggested mechanisms for the effect of diabetic condition on craniofacial complex

Growth of the craniofacial complex is controlled by genetic and environmental factors (Giglio and Lama, 2001; Yonemitsu *et al.*, 2007). Regulatory mechanisms responsible for normal morphogenesis of the face and head involve hormones, nutrients, mechanical forces, and various local growth factors. The poor growth and alterations in bone metabolism have been associated with T1DM in both humans and experimental animals (Giglio and Lama, 2001). Because human studies can be limited by small sample sizes, cross-sectional designs, uncontrolled variables, and often retrospective nature, animal models have been used to yield more rigorous analyses (Singleton *et al.*, 2006). In our studies we observed the rat growth from the age of 3 weeks old till 7 weeks old. This time period is corresponding to early growth stage in human according to previous craniofacial growth studies (Losken *et al.*, 1994; Siegel and Mooney, 1990). Consequently, in the current study STZ-DM model was used to investigate the effect of T1DM on craniofacial growth.

The studied STZ-DM rats showed significantly reduced growth in most of the craniofacial skeletal units but no significant differences were observed between controls and DM group as regards the remaining craniofacial skeletal units (Sphenoid bone length, posterior neurocranium height, anterior corpus length, bigonial width and palatal width). Craniofacial growth as a whole was also significantly lower in DM group compared to controls in all three dimensions. Previous study investigated the DM effect exclusively on the growth of the mandible and suggested that the diabetic condition had a differential effect on the osseous components and / or its associated non-skeletal tissues. They found that the disharmonious growth of the mandible was due to DM condition and might not be associated with diabetic condition complications such as renal failure, anemia, body weight change or alteration in the food intake qualities (Giglio and Lama, 2001). Thus we hypothesize that the deficiency in the craniofacial growth in our experiment might be attributed to the diabetic condition in the DM group as it was reported that specific alterations in bone metabolism are associated with DM. Moreover, several pathogenic possibilities have been proposed, such as insulinopenia, bone microangiopathy, impaired regulation of mineral metabolism, alterations in local factors that regulate bone remodeling, and even an intrinsic disorder associated with T1DM (Duarte *et al.*, 2005; Ward *et al.*, 2001). The aforementioned insulin hormone deficiency that is associated with T1DM cases may have direct effect on bone metabolism. It was mentioned in literature that normal insulin hormone level exerts direct anabolic effects on bone cells (Duarte *et al.*, 2005). Multiple osteoblast-like cell lines express insulin receptors on the cell surface and have a high capacity for insulin binding (Pun *et al.*, 1989). Moreover, osteoclasts exhibit reduced bone resorption in response to insulin stimulation (Thomas *et al.*, 1998). These findings support the idea that the actions of insulin in bone could be mediated directly via stimulation of osteoblasts in combination with inhibition of osteoclasts, (Thomas *et al.*, 1998; Thrailkill *et al.*,

2005) and this mechanism of action may explain the retardation of craniofacial growth in STZ-DM.

Diabetes has a deleterious effect on osseous turnover due to decreased osteoblast and osteoclast activities and numbers and, a lower percentage of osteoid surface and osteocalcin synthesis, as well as increased time for mineralization of osteoid (Duarte *et al*., 2005). It was reported that the influence of diabetes on discrete stages of matrix-induced endochondral bone formation could have profound effects on the biomechanical behavior of bone. Also, chondrogenesis and calcification of bone were reduced by 50% in diabetic animals (Reddy *et al*., 2001). This was evident in the current study results that showed a significant decrease in the craniofacial linear measurements of the DM group.

In addition to this, insulin may exert synergistic effects with other anabolic agents in bone, such as parathyroid hormone (PTH) (Thomas *et al*., 1998; Thrailkill *et al*., 2005). An animal model of T1DM has frequently demonstrated alteration in bone turnover, retarded growth, increased concentration of PTH, and reduced concentration of 1,25-dihydroxivitamin D (Duarte *et al*., 2005; Tsuchida *et al*., 2000). The effects of PTH on the bones are complex; it stimulates resorption or bone formation depending on the concentration used, the duration of the exhibition, and the administration method (Duarte *et al*., 2005; Toromanoff *et al*., 1997; Tsuchida *et al*., 2000). Also, 1,25-dihydroxivitamin D, like PTH, belongs to the most important group of bone regulatory hormones. It regulates osteoclastic differentiation from hematopoietic mononuclear cells, and osteoblastic functions and activity (Collins *et al*., 1998; Duarte *et al*., 2005).

Moreover, Insulin may indirectly regulate the enhancement of growth hormone serum concentration by direct regulation of the hepatic growth hormone receptor, this results in abnormalities in the insulin growth factor-1 in T1DM (Chiarelli *et al*., 2004) which consequently may have lead to the retarded growth in uncontrolled DM in the current study.

In the present study most of the periosteal surfaces in the mandibular regions of the control group showed significantly higher values recorded for the mineral apposition rate and the bone formation rate when compared to the DM group. These results agree with previous studies that recorded diminished lamellar bone formation in DM rats' femur and may suggest an association between the DM condition and the decreased number and function of osteoblasts (Follak *et al*., 2004; Shyng *et al*., 2001). The alveolar crest region was the only region that did not show a significant difference in the mineral apposition rate and the bone formation rate parameters among the two groups; this may be attributed to the unique nature of this region exhibiting a highly intensive bone remodeling process especially during the teeth eruption that decreases toward the base of the socket (Gerlach *et al*., 2002).

Micro CT analysis showed a significant decrease of bone volume fraction, trabecular thickness, and trabecular number. Also, it showed a significant increase of the trabecular separation and the trabecular space in the DM group when compared with the control group. This finding indicates deterioration of the bone quality in the DM group. These results agree with other research work suggesting that the glycaemic levels play an important role in modulating the trabecular architecture especially in mandibular bone (Thrailkill *et al*., 2005). In this context, these results may describe a state of osteopenia in experimental diabetic rats, which might be the result of an imbalance between bone formation and resorption.

A histometric evaluation of bone resorption was performed by counting the number of osteoclast cells on the distal surface of the alveolar bone adjacent to the mesio-buccal root of the second molar. These evaluations revealed that the number of osteoclasts was significantly lower in the DM rats than in the controls, in line with previous studies on DM rats' mandible (Mishima *et al.*, 2002) and long bones (Glajchen *et al.*, 1988; Shires *et al.*, 1981). These studies confirm that the decreased rate of bone turnover may be associated with the DM condition.

This deteriorating effect on mandibular bone structure and dynamic bone formation might be be attributed to several pathogenic possibilities, such as insulinopenia, bone microangiopathy, impaired regulation of mineral metabolism, alterations in local factors that regulate bone remodeling, and even an intrinsic disorder associated with DM (Abbassy *et al.*, 2008; Duarte *et al.*, 2005). However, the detrimental effects observed may not be associated with the significant loss of rats` weights observed in the diabetic group starting from day 14 because previous research (Abbassy *et al.*, 2008; Giglio and Lama, 2001; Shires *et al.*, 1981; Thrailkill *et al.*, 2005) showed that the mandibular growth was not affected in normal rats supplied with restricted diet and having same pattern of weight loss resembling weight loss pattern observed in DM rats.

8. Conclusions

It is obvious that the T1DM condition significantly affects craniofacial growth, bone formation mechanism and the quality of the bone formed which may alter many aspects of planning and treatment of orthodontic patients affected by this globally increasing hormonal disturbance.

There should be a new strategy for treating orthodontic patients suffering from metabolic disorders specially those disorders having direct and indirect effects on bone growth as the diabetic condition. The orthodontic craniofacial linear measurements were significantly decreased in the T1DM cases when compared to normal cases. These facts should be considered when orthodontic problems are diagnosed and treated in T1DM, because this may alter the orthodontic treatment period and the final treatment outcome. In this chapter it was suggested that a better understanding of how diabetes affects bone will improve our ability to protect bone health in diabetic patients. Also, it was recommended to conduct further studies in order to elucidate the exact mechanism by which diabetes produces alterations in most of the craniofacial units.

Thus, T1DM condition had detrimental effects on the different bone regions of the mandible, that shed the light on the complexity of the craniofacial structure which is affected by many hormonal disorders suggesting further examination for the overlying soft tissues which are directly affected by any underlying boney changes. Diabetes condition had detrimental effect on bone architecture, as shown by micro-CT, and impaired the rate of the mandibular bone formation, as examined by the dynamic histomorphometric analysis. All of these results were verified on the cellular level by a histological study that showed the diminished number of osteoclasts on the alveolar wall, suggesting that the early stage of T1DM resulted in low bone turnover. These results may suggest that more care is needed during the consideration of the force needed for treatment of orthodontic patients affected by T1DM, also, the retention period and rate of success in these patients may be affected.

These comprehensive studies done on bone and craniofacial growth suggest that planning the treatment in craniofacial region for patients affected with hormonal disorders is a more

complex procedure when compared to the treatment of normal patients, moreover it is suggested that it is of prime importance to keep close attention to the general systemic condition of these patients and administer the proper hormonal therapy for these patients when needed to avoid any detrimental effects on bone resulting from any hormonal imbalance.

Within the limitations of this *in vitro* study, it was concluded that:

1. T1DM reduces craniofacial growth, resulting in retardation of skeletal development.
2. DM in the rat affects the bone architecture, as shown by micro-CT, and impairs the rate of the mandibular bone formation, as examined by the dynamic histomorphometric analysis.
3. All of these results were verified on the cellular level by a histological study that showed the diminished number of osteoclasts on the alveolar wall, suggesting that the early stage of DM resulted in low bone turnover.
4. These findings should be considered when conducting any treatment in the craniofacial region in T1DM since the better understanding of how diabetes affects bone will improve our ability to protect bone health in diabetic patients.
5. Good control of diabetes mellitus should be considered before orthodontic treatment by a long time inorder to obtain the best outcome.

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22

The Role of Genetic Predisposition in Diagnosis and Therapy of Periodontal Diseases in Type 1 Diabetes Mellitus

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1. Introduction

The prevalence of diabetes mellitus (DM) and its probable influence on periodontal disease suggests that DM patients will very likely probably become an increasing proportion of the patient population seen by both general dentists and periodontists. Many investigators have studied the oral manifestations involving periodontitis as a complicating factor in the periodontal therapy of DM patients, whose disease may be more prevalent and more severe and progress rapidly.[1] [2] [3] Periodontal disease makes chewing difficult or painful, thereby leading to an improper diet. On the other hand, uncontrolled periodontal disease may upset metabolic control of DM.[2]

Until the past 15 years, the management was based on the periodontology model of care, and the aim of these methods was to diagnose the problem and resolve it via treatment. Consequently, repairs were made, but the periodontitis generally recurred or progressed unabated. Disease prevention was not practised. It is clear that the risk of periodontal disease varies greatly from one patient to another.[4-8]

Today many practitioners are providing better and more complete service to their patients because they are beginning to incorporate the principles of the information-intense medical model.[9] Changes in our political and social structure have affected how health care is being managed. With increasing evidence of the influence of periodontal disease on systemic health, dentist and hygienists are taking a more intensive look at the risk factors associated with its onset and progression. Bacteriology, immunology, genetics, and systemic cofactors are often used to determine what is wrong with the patients. Genetic knowledge is an important part of the medical model because it allows for a complete and comprehensive picture of all of the factors contributing to the patient's past, current, and future status.

2. Classification and characterization of periodontal diseases

2.1 Gingivitis

Gingivitis is an inflammatory pathologic alteration affecting the gingival epithelium and connective tissue (Fig.1). Clinical symptoms comprise reddness and swelling of the gingiva, bleeding on probing, and a periodontal pocket deph ≥1mm.

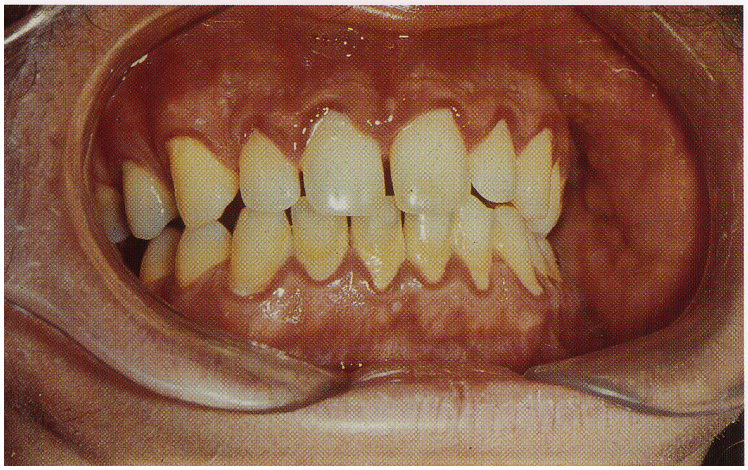


Fig. 1. Gingivitis

Gigivitis is characterized by a subgingival microflora that is slightly shifted in favor of gramm-negativ, anaerobic bacteria without any periodontopathogenic microorganisms. ANUG is the acronym of the acute necrotic ulcerative gingivitis.

2.2 Chronic Adult Periodontitis (AP)

The slowly progressing AP is clinically characterized by persistent loss of attachment, a positive bleeding on probing (BOP), periodontal pockets depths of 1-3 mm, and slight loss of alveolar bone tissue (Fig.2). This is the most common type of periodontitis and generally occurs at the age of 30 years or later. It may be either generalized or limited to molars and/or incisors. The subgingival microbial panel is slightly shifted in favor of gram-negative, anaerobic bacteria with an increased amount of periopathogenic microorganizms.

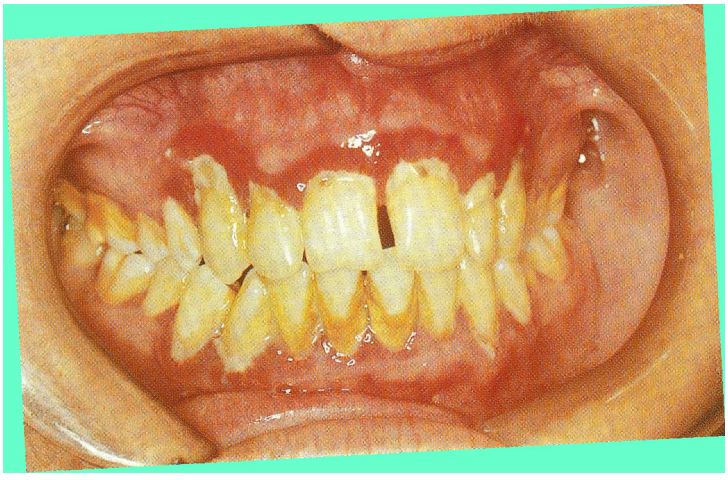


Fig. 2. Periodontitis (loss of alveolar bone tissue)

2.3 Refractory marginal periodontitis

Refractory or therapy-resistant AP shows a progressive loss of attachment even with diligent mechanical therapy and positive compliance of the patients. This type of periodontitis is characterized by a progressive loss of supporting tissue even if treated thoroughly. In most cases the lesions compromise more than one tooth and are infected by high concentration s of periodontal pathogens. After a massive degradation of supporting tissue the affected teeth are often lost (Fig.3).

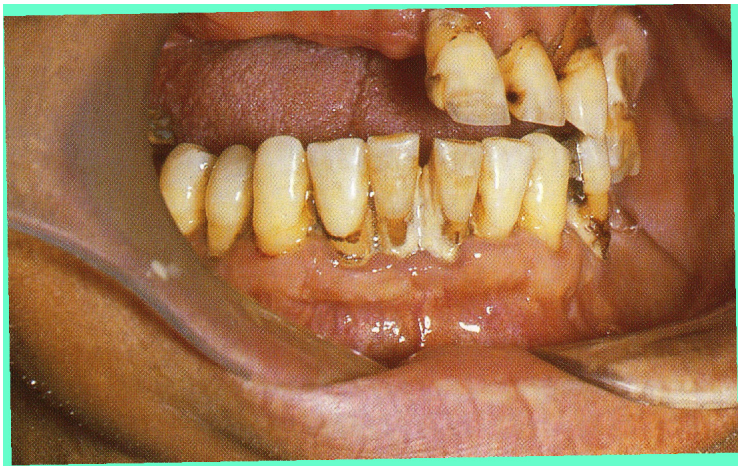


Fig. 3. Periodontitis (degradation of supporting tissue the affected teeth are often lost)

2.4 Localised Juvenile Periodontitis (LJP)

This type usually affects healthy adolescents at the age of 10 to 20 years. It is distinguished by a severe, but localized loss of bone tissue combined with the development of deep pockets at the first molars and/or the incisors. *Actinobacillus actinomycetemcomitans* is considered to be the marker pathogen for LJP.

2.5 Rapidly Progressive Periodontitis (RP)

RPP is mostly seen in patient's at the age of 20 to 35 years. The patients medical history frequently shows a prior LJP. This form of periodontitis is generalized and progresses intermittently with severe loss of bone tissue, gingival bleeding and acute inflammation.The subgingival oral microflora is characterized by a high concentration of periodontal pathogens.

3. The oral cavity ecosystem

The human oral cavity accomodates about 50 billion bacteria belonging to about 400 different species.[10] The various habitats are occupied by microbial populations specifically adapted to their enviroments.

The deep periodontal lesion is a unique eco-system within the oral cavity providing particular living conditions. It is the only place within the oral cavity not being flushed by saliva. Instead is filled with crevicular fluid. Moreover, the acigen concentration decreases progressively with pocket depth creating optimal growth conditions for anaerobic bacteria.[1]

These bacteria lack the enzymes necessary to detoxify oxigen radicals resulting in a severely reduced growth or even death in the presence of oxigen. Dispite the great variety of the microbial flora only a fraction of the bacterial species is etiologically connected to the development of periodontitis. The flora of a healthy sulcus usually consists of aerobic grampositive cocci and rods. These bacteria known as „beneficial flora" show no pathogenic potential but, by their presence, are able to prevent the colonization is progressing the microbial spectrum is shifted in favor of anaerobic gram-negative rods.
The marker pathogens of periodontitis belong to the group of obligatory anaerobic, black pigmented Bacteroides species as *Porphyromonas gingivalis* and *Prevotella intermedia* as well as *Bacteroides forsythus, Treponema denticola, and Actinobacillus actinomycetemcomitans*.[11,12] A strict correlation of the alveolar pocket depth and presence of periodontopathogens could be proven by several clinical studies.[13,14,15]
Several metabolites produced by the periodontitis-associated pathogens either destroy the sorrounding periodontal tissue or inactivate the humoral host defense system. The most important virulence factors produced by the three marker pathogens *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans*.[14]

4. Aetiology of periodontitis

The development of plaque is considered to be the primary cause of periodontitis. Especially at the gingival margin plaque hardens to tartar resulting in a mechanical irritation. Exotoxins produced by the plaque bacteria diffuse into adjacent tissue and give rise to reddening and swelling -the typical clinical characteritics- of gingivitis. After professional removal of plaque and dental calculus a healthy periodontium is quickly restored indicating that gingivitis is a reversible condition. In case no professional removal of the dental calculus is performed the infection will progress and a periodontitis becomes established showing the typical clinical characteristics like bleeding on probing (BOP), increasing pocket deph and loss of alveolare bone tissue.[11]
At the age play a roule development of periodontitis[16], and the age of 40 far more teeth are lost by periodontitis than by caries.
Periodontitis associated bacteria are found in low concentration even in the healthy sulcus. Therefore, additional factors must exist that determine the onset and the progress of disease. In case of an impaired immune system e.g. by stress, medication, hormonal imbalances, diabetes or smoking the pathogens utilize the selective advantages for prompt proliferation leading to the establishment of an manifest infection of the periodontium.[17-20]

5. Genetic component to periodontitis

For approximately the past 15 years, dental researchers have been focusing on dental plaque. Clinicians have made treatment decisions as though the plaque-disease interrelationship was quantitative: the more plaque, the more bacteria, the more inflammation, the more disease. However, clinical experience demonstrates that not all people respond the same way to similar accumulations of plaque. There are patients with a lot of plaque who have moderate and advanced disease. Some types of plaque simply are more virulent than others. However, clinical experience demonstrates that not all people respond the same way to plaque accumulations. Because so much variability respond to plaque, and respond to treatment. Some type of plaque symply are more virulent than

others. In adults, these bacteria routinely colonize the teeth when tooth cleaning is not performed on a regular basis. Although bacteria are essential for the initiation of periodontitis, there is currently no mechanism for determing the clinical trajectory of the disease for individual patients, i.e., differentiating those patients who will have a mild to moderate form of disease and respond well to simple professional care from those who are likely to develop a more severe periodontitis that demands extensive therapy and results in tooth mobility. Individual differences in disease progression are dramatic and are often not predictable by currently known mechanisms.

Identification of a risk factors may explain why individual patients do not respond uniformly to standard treatment. For example, a patient who is a heavy smoker may not heal as soon or as well as expected after treatment. Patients react differently to bacterial stimulation. This is a result not only of the type and amount of bacteria but also of the underlying genetic characteristics of the patient's immune system.The cytokines tumor necrosis factor alpha(TNFά) and interleukin 1 (IL-1) are key mediators of the inflammatory process and modulate the extracellular matrix components and bone which comprise the periodontal tissues.The genes encode pro-inflammatory proteins (IL 1A and IL-1B producing IL-1 ά and IL-1β). Several genetic polimorphisms have been described in the genes of the IL-1 cluster and, in case control studies, associations have been reported with increased severity of several chronic inflammatory diseases.

The genetic risk of developing periodontitis has been investigated by studying families and populations as well as twin.[21-26] The studies conducted on twins have reported a significant genetic component explaining the variation in clinical attachment loss, probing depth and gingivitis. An association between the severe chronic form of the disease and a composite genotype in the interleukin IL-1ά and IL-1β genes has been reported.[21] However, this association was found only in non-smokers. Other authors have subsequently investigated the IL-1ά - IL-1β genotypes with a chronic form of periodontitis with different results.[27,28]

The genetics influence resistance on periodontal disease has been determined from a wide variety of sources.[29-32] Genotype positive patients had significantly more clinical expression of inflammation, as determined by bleeding on probing. In healthy patients 46.7 % of genotype positive patients had bleeding as compared with only 8.6% of genotype-negative patients.[32,33] Their results from genetic susceptibility testing have the real potential to improve patient management. Many of the genetic markers for common disease involve polymorphisms in gene sequences involved in cytokine biological activity. Researchers know that in healthy subject IL-1 plays a very important role in inflammation and the expression of periodontitis. Patients with this genotype progress more rapidly toward severe periodontitis and have statistically significant increased inflammation.[32] It has been established that this genotype occurs in approximately 30% of most of the populations that have been tested for this genotype.

Cells from people with a positive genotype produced up to four times more IL-1 in response to the same bacterial challenge.[34] Because IL-1 in high concentrations is involved with destruction of tissues, this increased IL-1 response may explain the more rapid progression of periodontal disease in genotype-positive patients when faced with a bacterial challenge in their plaque. For patients with this genetic susceptibility to periodontitis, tooth loss was be minimized by good plaque control and definitive periodontal therapy.[35]

6. Genetic test for susceptibility to periodontal disease

Two polymorphisms within the IL-1 gene cluster show a close association with periodontitis. One polymorphism is located at position -899 of the Interleukin 1ά gene, the other at position +3953 of the Interleukin 1β gene.[32,36] Within both polymorphisms allele 1 harbors a cytidin c, whereas allele 2 carries a thymidin (T) at the respective position. Allele 2 of the +3953 polymorphism of the IL-1β gene leads to an alteration of the corresponding protein resulting in an overproduction of IL-1β.[37] This overproduction of IL-1 seems to override the feedback mechanisms which normally limit inflammation resulting in the development of massive gingival pockets and degradation of periodontal tissue. These data allow a risk assessment, defining a patients as PRT-positive or PRT negative, the presence of periodontitis risk alleles at positions IL-1ά-889 and IL-1β +3953.

6.1 Genotyp^{R} PRT test

With the Genotyp^{R} PRT test (Hain Lifescience) the base composition and allelic combination of the two IL1 loci can be analysed. The test is a molecular biological assay based on the identification of gene loci associated with an elevated risk in developing periodontitis by means of highly specific DNA probes. It is based on the analysis of nucleic acids, there is no need for viable bacteria to perform the test and no special precautions are required during transport. A detailed sequence analysis has to be performed by additional examinations. The Genotyp^{R} PRT test is not a diagnostic test for periodontal disease. It is rather a test determing the patient's genetic susceptibility to developing severe, generalized periodontitis in the future and helps to plan a comprehensive therapy. Processing and interpretation of the test is performed in clinical laboratories from a buccal swab containing cells of the mucous membrane of the patient's cheek.

7. Indications for microbiological testing of the subgingival flora

It is generally accepted that periodontitis is initiated by the establishment of a specific subgingival bacterial flora. Some of the marker pathogens belong to the group of obligatory anaerobic, black pigmented *Bacteroides species* such as *Porphyromonas gingivalis* and *Prevotella intermedia.* In addition, the bacterial species *Actinobacillus actinomycetemcomitans* (*Haemophilus a.*), *Bacteroidea forsythus,* and *Treponema denticola* play a pivotal role in the initiation of periodontal disease (Table 1).

Strong evidence for etiology	Moderate evidence for etiology
Actinobacillus actinomycetemcomitans	Campylobacter rectus
Porphyromonas gingivalis	Eubacterium nodatum
Bacteroides forsytus	Fusobacterium nucleatum
	Prevotella intermedia
	Peptostreptococcus micros
	Streptococcus intermedius-complex
	Treponema denticola

Table 1. Specific Bacteria Associated with Periodontal Disease (Annals of Periodontology 1:928,1996)

7.1 MicroDent^R test

Like Genotyp^R PRT the microDent^R test is a molecular biological diagnostic device. Since it is based on the analysis of nucleic acids, there is no need for viable bacteria to perform the test and no special precautions are requireed during transport. The microDent^R test a highly sensitive and highly specific molecular bological PCR-DNA-probe method. Due to the high specificity of the PCR, any potential contamination of the probe by concomitant flora has no influence on the test results.

A defined cut-off ensures that every positive test result is of clinical relevance and that bacterial concentrations present in a healthy sulcus lead to a negative result. Sampling is performed from the gingival pocket with strile paper points. These marker species can be detected with the microDent^R test: *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Bacteroides forsythus, Treponema denticola.*

Periodontopathogenic bacteria activate inflammatory mechanisms within the local periodontal tissue throught the production of toxins and other metabolites. The degree of this response depens on the general health and immunologic state of the patients. Besides that, exogenic risk factors such as haevy smoking, stress and medication can negatively influence the progression of periodontal disease. Patients who in addition are PRT-positive suffer from an overproduction of IL-1 leading to a significantly increased immunologic response to the presence of periodontopathogenic bacteria. This individuals therefore are at an even higher risk for developing severe disease and losing teeth. Knowledge of the IL—1 genotype, the bacterial load, and possible additional risk factors allow for the prediction of the patient's future periodontal status including the risk of further tooth loss.

8. Risk factor influence on periodontitis in type 1 DM

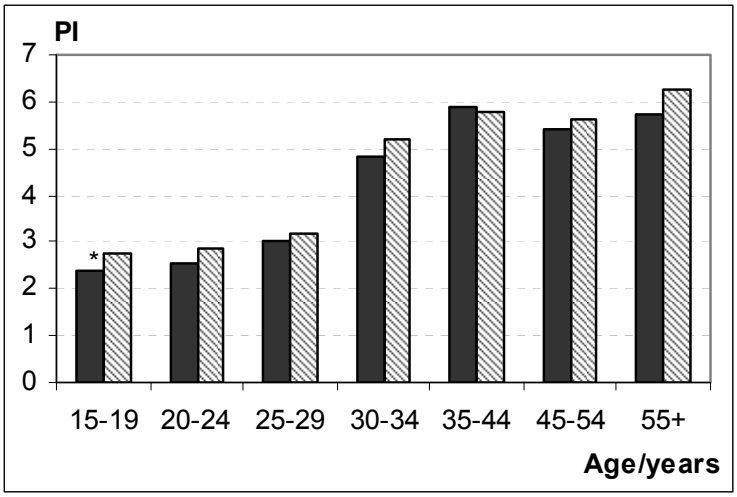
A number of studies have demonstrated a relationship between DM and periodontal diseases, which are among the most prevalent complications of DM.[38-57] Individuals with DM tend to have a higher prevalence of periodontal diseases and more severe and rapidly progressing forms than those who do not have DM.[41,48] DM is a known risk factor for periodontitis in adults. Seppälä et al.[49] demonstrated that patients with type 1 DM exhibit a higher degree of attachment loss and bone loss than control subjects under similar dental plaque conditions. This finding was confirmed in a follow-up site-by-site study by the same authors.[50]

The changes in the periodontal conditions are mostly expressed in the first year of the disease, and the damage to the periodontium which develops at this time is not greatly influeced in the further course of the disease (Fig 4).

It is an interesting result that younger DM subjects display more periodontal destruction than do non-DM subjects at a later age. In the all-age groups, the periodontal status varied according to the age of the patient at the onset of DM. This suggests that the early onset of DM (before 14) is a much greater risk factor for periodontal diseases than mere disease duration (Fig 5).

Earlier investigators[51,52] noted that the duration of DM was greater in groups with severe periodontal disease. Our results[53] indicated that DM is associated with an increased risk of the development of periodontal disease in the event of an increased duration of the DM, and the level of oral hygiene is considered to be a contributory factor rather than the primary etiologic factor in the initiation of gingivitis and periodontitis in those with DM. In agreement with Gusberti et al.[57] the findings of our study[53] demonstrate that poorly controlled type 1 DM patients with elevated blood glucose and HbA_{1c} levels have a greater

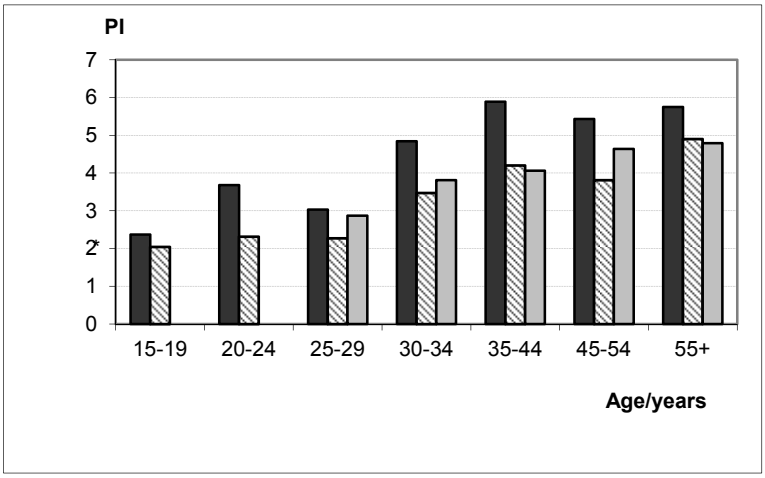
prevalence to more severe periodontal diseases. The severity of periodontal disease was observed to decrease as the control of the DM improved, in agreement with Tervonen and Knuuttila[55], Rylander et al.[58]



Duration of DM: < 1year / > 1year: *P<0.001

Duration of DM: ■ <1 year, □ >1 year

Fig. 4. Periodontal conditions (PI) in patients with short and long histories of DM.

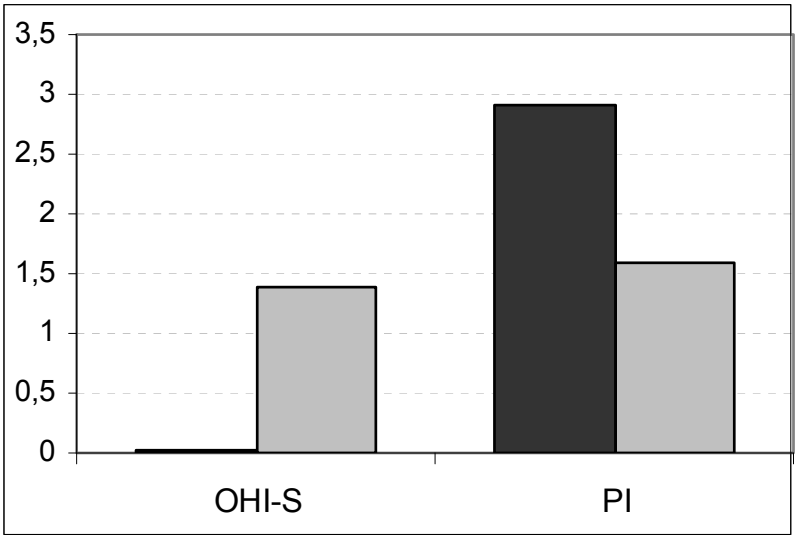


< 14 year / > 14 year: P<0.0001

■ < 14 year, ▨ 15-25 year, □ > 25 year

Fig. 5. Age of the patient at the onset of DM

Type 1 DM to increase the prevalence and severity of periodontitis independent of the effects of oral hygiene, and duration time of DM.[42] However the severity of periodontal disease increased with the duration of DM only among those with an adequate level of oral hygiene (OHI-S = 0) The association between periodontal disease and the duration of diabetes mellitus is consistent with trends seen in other complications of DM whereas the longer duration of diabetes mellitus is in direct proportion of the prevalence and severity of periodontal disease. The development of systemic complications of diabetes such as retinopathy, nephropathy, is also is relationship with the duration of diabetes mellitus agreement with Rylander[58], Galea et al.[59], Rosenthal et al.[60] and Lopez.[52]



PI = The intensity of gingivitis and periodontitis
OHI-S = oral hygiene
OHI-S= 0 / PI: p<0.0001
Duration time: ■ < 1 year □ > 5 year

Fig. 6. The intensity of gingivitis and periodontitis(PI) according to the level of oral hygiene (OHI-S) and the duration of DM

On the other hand, the presence of severe periodontal infection may also increase the risk for microvascular and macrovascular complications. DM patients with severe periodontal disease demonstrate a significantly higher prevalence of proteinuria and a greater number of cardiovascular complications.[31,54,55] Karjalainen et al.[56], Genco,[31] Lopez et al.[52], Albrecht[59] examined the association between the severity of periodontal disease and organ complications (retinopathy) and found that advanced periodontal disease was associated with severe ophthalmic complications in type 1 DM.
A more pronounced incidence of poor glycemic control in subjects with a shorter duration of DM would be consistent with the hypothesis that hyperglycemia increases linearly with time, but at different rates in different people. This hypothesis suggests that patients with

rapidly increasing hyperglycemia would have more severe periodontal disease at the onset of DM, resulting in damage to the periodontium.

A positive correlation between the level of control of the disease and the intensity of gingivitis and periodontitis. In the well-controlled type 1 DM patients the intensity of gingivitis and periodontitis was lower than in those with poor glycemic control agreement with Gusberti et al.[57], Albrecht et al.[41] Good metabolic control of DM reduces the susceptibility to infection and is therefore also important for the prevention of periodontal disease in people with type 1 DM. In patients with poorly controlled DM, an improvement of the metabolic control may improve the periodontal condition.[61-64] Conversely, periodontal disease can interfere with the control of DM and can increase the insulin requirements in previously stable patients.[65-67,56,68,2,69]

Smoking is associated with an increased intensity of periodontitis. Very light or occasional smokers did not show statistically significant differences compared to non-smokers respect to the prevalence and intensity of gingivitis or periodontitis. No periodontally healthy subjects who has been, or who use to be heavy smokers. Tobacco contains cytotoxic substances such as nicotine which may also have a negative effect on the cellular turnover and repair of the periodontium.[68,70,53]

9. Genetic predisposition and periodontal disease in type 1 DM

Recently several study demonstrates that specific genetic markers, that have been associated with increased IL-1 production, are a strong indicator of susceptibility to severe periodontitis in healthy adults. [22,29,31] The study presented here was to explore a possible association between IL-1A and IL-1-B genotypes in patient with type 1 DM and controls with periodontitis. The frequency -in type 1 DM and controls- of the composite genotype that comprises allele 2 of the IL-1A plus IL-1B is shown in Fig.7. All subject were non-smokers.

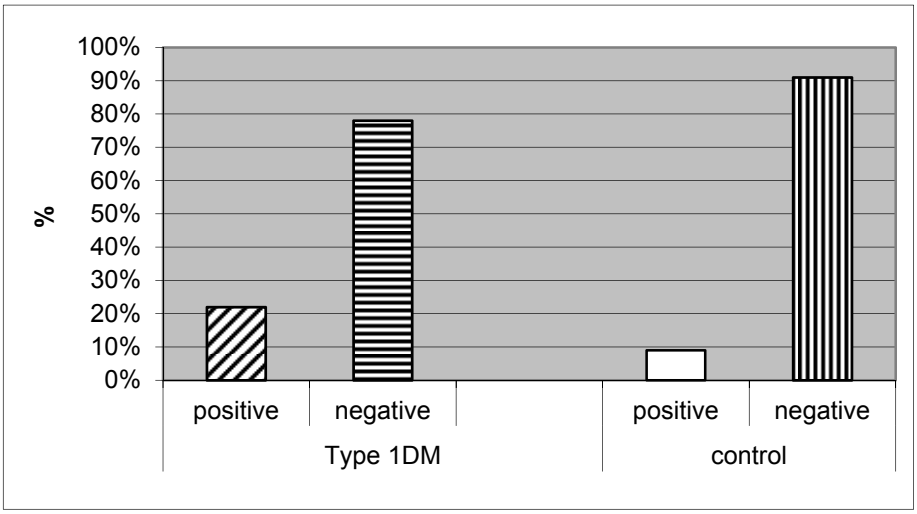


Fig. 7. Frequency of the Genotype[R] PRT positive and negative type 1 DM adult patients and control with periodontitis.

To control the effect of age on disease severity, data were analyzed separately for type 1 DM adolescents aged 14-19 years. In this age range, the composite genotype was present in 22,7 % of DM adolescents and 8,57% of healthy individuals were estimated to carry the IL-1 risk genotype. Distribution of the PAG (Periodontitis Associated Genotype)[21] positive and negative subjects in type 1 DM adolescents shows the Fig. 8.

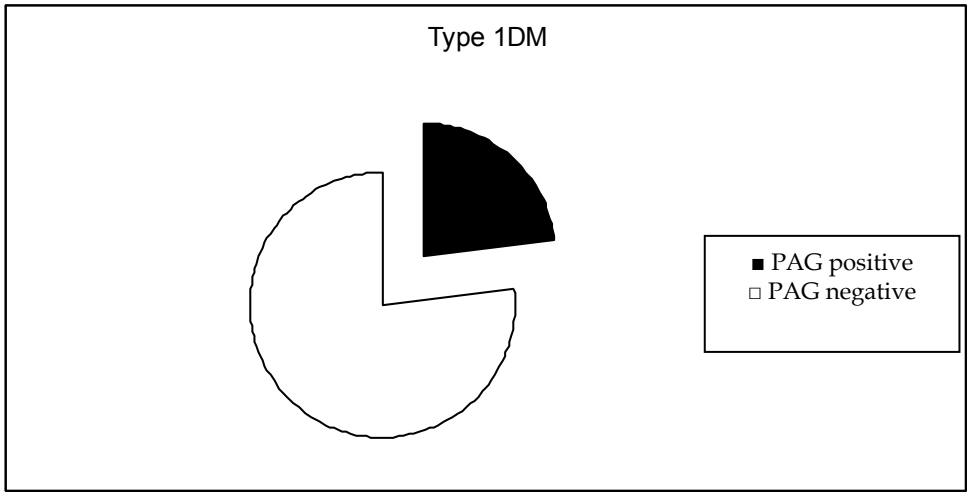
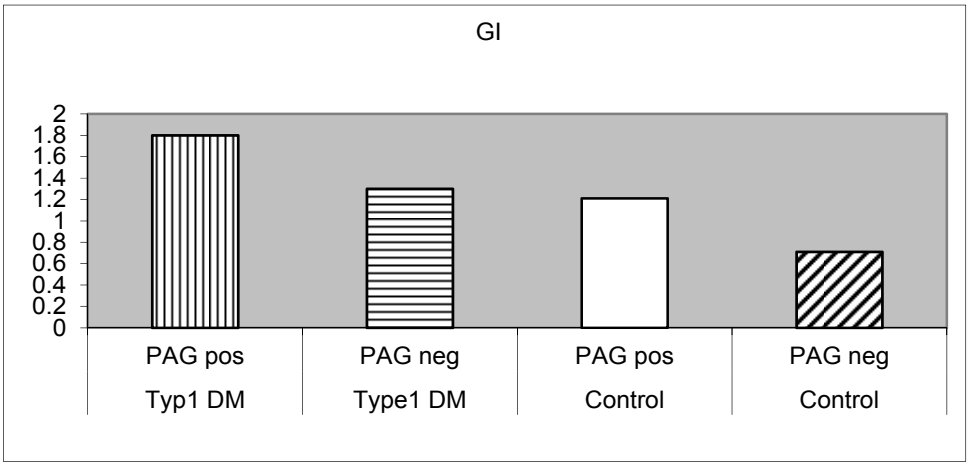


Fig. 8. Frequency of PAG positive and negative adolescents with type 1 DM

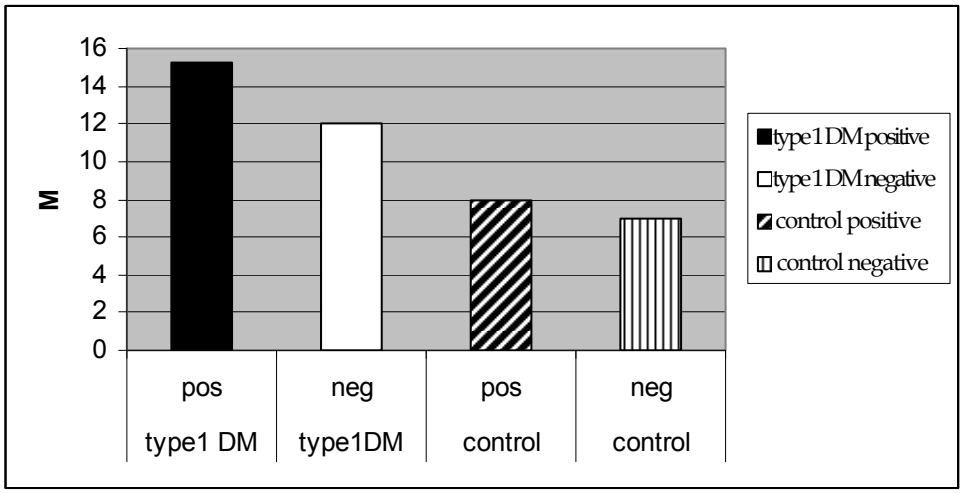
Gingivitis was more severe in those adolescents with positive GenoType^{R}PRT test (Fig.9).



PAG positive/PAG negative: $p<0.001$

Fig. 9. The intensity of gingivitis (GI) of PAG positiv and in negative adolescents with type 1 DM.

In type 1 DM there was significant more extracted teeth in Genotype^R PRT positive subjects than in negative group or non-diabetic people ($p< 0.001$) (Fig.10).



P< 0.001

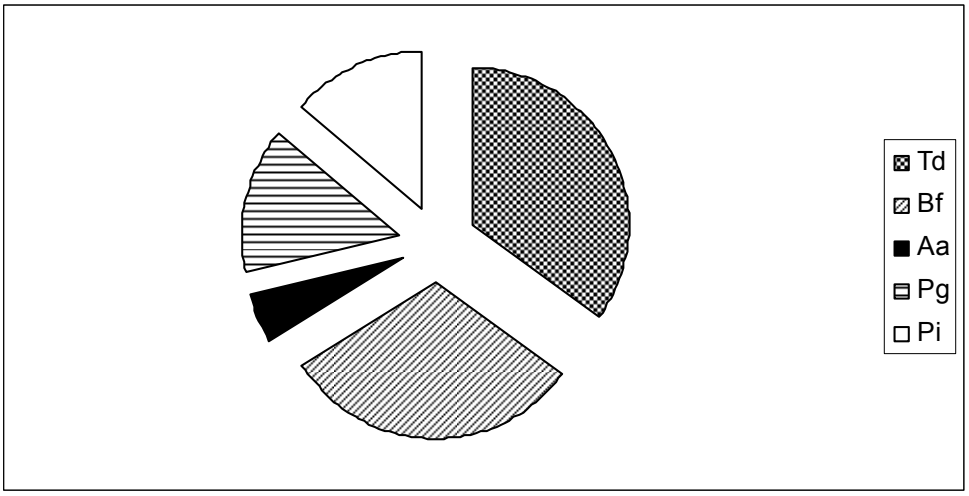
Fig. 10. The mean value of extracted teeth (M) in DM patients and metabolically healthy individuals with positive Genotype.

Periodontitis involves multiple clinical patterns including various severities of periodontitis, uncommon early onset forms that affect children and young adults with type 1 DM, and patients who do not respond predictably to conventional therapy refractory periodontitis.
Guzman et al.[71] have shown a possible interactions between genetic an enviromental factors that there is interplay between genetic an environmental factors that results in periodontal disease.
The finding that a specific genotype in the IL-1 gene cluster correlates with severe periodontitis suggest a genetic mechanism by which some individuals, if challenged by bacterial accumulations, may have a more vigorous immuno-inflammatory response leading to more severe periodontitis in type 1 DM. The lack of reliable markers for type 1 DM patient susceptibility to severe periodontitis has prevented the early identification of those at most risk and has prevented delivery of therapy appropriate for the degree of the risk.

10. The modification of the subgingival microflora in patients with type 1 DM

Periodontopathogenic bacteria activate inflammatory mechanisms within the local periodontal tissue throught the production of toxins and other metabolites. The degree of this response depends on the general health and immunologic state of the patients. Besides that, exogenic risk factors such as heavy smoking, stress, and medication can negatively influence the progression of periodontal disease. Of all of the various microorganism that colonize the mouth in healthy subjects, there are three, Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, and Bacteroides forsythus have been implicated as etiologic agents in periodontitis. The presence of periodontal pathogen, though necessary to

cause disease, is not sufficient. According our findings there were a significant difference between the severity of the gingivitis, parodontitis and the bacteria identified from the gingival pocket of patients with type 1 DM (Fig.11, Table 2). In case of gingivitis alone, the most prevalent bacterium was *Bacteroides forsythus* (11.11%), whereas in parodontitis it was *Treponema denticola* (75.92%). In type 1 DM the presence of *Treponema denticola* no additional risk of developing agressive periodontitis, despite the fact its presence is necessary for the disease to develop. The *Bacteriodes forsythus, Prevotella intermedia, Porphyromonas gingivalis, Actinobacillus actinomicetemcomitans* may be risk indicators for periodontal disease in population with type 1DM though they are not risk factors.
Patients who in addition are PRT positive suffer from an overproduction of IL-1 leading to a significantly increased immunologic response to the presence of periodontopathogenic bacteria. These individuals therefore are at an even higher risk for developing severe disease and losing teeth.



Td: *Treponema denticola*
Bf: *Bacteroides forsythus*
Aa: *Actinobacillus actinomycetemcomitans*
Pg: *Porphyromonas gingivalis*

Fig. 11. The distribution of the subgingival microflora in adult patient with type 1 DM.

Subgingival bacterial flora in type 1 DM

Actinobacillus actinomycetemcomitans(Aa)	Porphyromonas gingivalis (Pg)	Prevotella inermedia(Pi)	Bacteroides forsythus (Bf)	Treponema denticola (Td)
5%	15%	14%	31%	35%

Table 2. Distribution of the Subgingival Microflora in Type 1 DM with Periodontal Disease

11. Diagnosis and therapy of periodontal disease in type 1 DM

A considerable fraction of periodontal diseases can be stabilized for years using classical mechanical treatments as root planing or deep scaling. However, this treatment often is not sufficient for elimination of the tissue invading periodontal pathogens. Subsequently, progressive loss of attachment and bone tissue might occur in spite of diligent treatment. In these cases a specific concomitant therapy with antibiotics promises to be more efficient -of course only after careful microbiological testing-.

The choice of medication and mode of application depends on the composition of the subgingival flora and the clinical manifestation of the periodontitis. Where tissue-invasive, periopathogenic bacteria such as *Treponema denticola* are present, mechanical methods like root-planing or deep-scaling are often ineffective in eliminating the pathogen. Despite careful treatment, the result is progressive attachment loss and bone resorption. In such cases, a one-of antimicrobial concomittal therapy – only undertaken after microbiological diagnostics, of course – is much more effective while causing less side effects.

In the main, both local and systemic antibiotic applications are available. In the case of a generalized periodontal disease, an adjuvant systemic therapy is indicated. If the infection focus is limited to individual sites, a local treatment is a sensible alternative.

Antibiotic therapies should in any case only be implemented after microbiological diagnostics (e.g.microDent^{R} test) have been completed, in order to avoid both excessive and under treatment.

In most cases a negative bacterium test result can be equated with periodontal stability. However, the presence of periodontal marker organisms indicates an increased risk for progressive destruction of the periodontium. It is obvious that a therapy with antibiotics should be initiated only after thorough microbiological test.

A sustained success of the therapy depends on an optimal compliance of the patients and regular recall sessions. Regular control examinations of the subgingival flora are rather helpful in early diagnosis of potential rezidives.

Genetics factors should also be determined because they play a key role in providing information to make better treatment decisions. A genotype result is always important to consider when making treatment decisions, even if the patient is genotype-negative. A negative result does not mean that a patient will be periodontal-disease-free. Genotype negative individuals must still be cautious about other risk factors, such as stress, smoking, bad oral hygiene-, and diabetes control.

The genetic predictive test for periodontitis complements the dentist's full scope of services by providing additional wanted and useful information. Indications for the genetic predictive test may be of value to the following patients groups:

- DM patients exhibiting refractory, therapy-resistent periodontitis. A positive test result might explain previous treatment failures and is an indicator for planing an alternative therapy.
- DM patients exhibiting progressive periodontitis. A positive test result might indicate the necessity for a more agressive therapy and shortes recall intervals.
- Type 1 DM adolescens -under 14 years- exhibiting early clinical sign of periodontitis. Before starting treatment, the test helps to plan an individual therapy maching the patient's t.i. a therapy stopping progress of the disease without risking over-treatment.
- Haewy smokers patients with type 1 DM.

Treatment decisions will be affected if a patient has risk factors, for example, haevy smokers. Haevy smokers are counseled that the treatment outcome from regenerative therapy will not be as good as outcomes for those who do not smoke.
In all cases where patient motivation and compliance are major obstacles to efficient prophylactic measures the situation can be dramatically improved when high risk patients are informed about their condition.
Knowledge of the IL-1 genotype, the bacterial load, and possible additional risk factors allow for the prediction of the patient's future periodontal status including the risk of further tooth loss. For the first time, these data enable the dentist to plan an individual therapy matching the patient's needs. Knowledge of the IL-1 genotype also allows a more efficient therapy from an economic point of view because over- and under-treatment can be minimized.

12. Conclusion

Periodontitis is a complex multifactorial disease. Similarly, Type 1 DM is a complex metabolic syndrome. Periodontal disease can be especially problematic for individuals with type 1 DM, in whom the disease may have an early onset or may progress more rapidly. Many characteristics and local factors such as dental calculus, the smoking habits and general factors (the duration of DM, the age, the degree of metabolic control, and the complications of DM) have been identified as factors that put people et an enhanced risk.
The initial dental therapy for patients with type 1 DM, as for all patients, must be directed toward the control of acute oral infections at the onset of DM. It is important to advise the phisician of the periodontal status, since the presence of infections including advanced periodontal disease may increase the insulin resistance and cotribute to a worsening of the DM state. Regular dental care may help maintain good oral health and it is especially important at the onset of the disease. Patients should also be checked regularly for bleeding gums or inflammation. Educating the patient in proper home oral care is a standard routine of periodontal treatment and prevention. Plaque control and scaling procedures frequently resolve gingivitis. However, where more tissue destruction has occurred, it may still be difficult or impossible for the patient to remove plaque deposits from the periodontal pockets.
Periodontitis is a disease leading to destruction of connective tissue and bone that support the teeth. Due to the multifunctional etiology of the disease its clinical appearence and progression greatly vary resulting in difficulties in planing an effective therapy. Smoking should be avoided because it is associated with an increased intensity of periodontitis.
Patients who are genotype positive do not lose all of their teeth to periodontitis because, for the most part, it is a treatable and preventable disease. If patients are identified as having the genetic susceptibility factor and begin a treatment or prevention plan, there are high expectations for favorable outcomes and there is no known risk from being tested. Determining the patient's genetic susceptibility in the future helps to plan a comprehensiv therapy and regular dental control to improve patients care prevent to developing severe, generalized periodontitis and toothlos. This IL-1 genotype does not cause periodontal disease directly, this marker is not a causative factor, instead it is a severity risk factor for susceptibility or predisposition to periodontitis. Optimal prophylaxis and efficient therapy of the polymorphisms defining the periodontitis risk genotype (PRT-positive) patients a careful microbiological testing of the subgingival bacterial flora for allows for the timely

application of appropriate therapeutic measures. In case of positive test results proving the presence of specific periodontopathogenic bacterial species antibiotics should be applied. Choice of medication and mode of application depend on the composition of the subgingival flora and the clinical manifestation of the periodontal disease.

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