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Treatment of Diabetes and Long-term Survival Following Insulin and Glucokinase Gene Therapy: a Proof-of-concept Study in Dogs

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BELLATERRA

A mi padre,
que le hubiera hecho
mucha ilusión haberla
visto acabada.

A mi madre
y a Mónica por su
incansable apoyo.

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AAV	Adeno-associated virus
AAV1	Adeno-associated vector serotype 1
AAV2	Adeno-associated vector serotype 2
AAV5	Adeno-associated vector serotype 5
AAV6	Adeno-associated vector serotype 6
AAV7	Adeno-associated vector serotype 7
AAV8	Adeno-associated vector serotype 8
AAV9	Adeno-associated vector serotype 9
AAV10	Adeno-associated vector serotype 10
AAV11	Adeno-associated vector serotype 11
AAV2RSS	AAV2 Reference Standard Material
AAV1-GFP	AAV1 vector that encodes for the GFP gene
AAV1-Gck	AAV1 vector that encodes for the Gck gene
AAV1-rGck	AAV1 vector that encodes for the rat Gck gene
AAV1-ohGck	AAV1 vector that encodes for the optimized human Gck gene
AAV1-Ins	AAV1 vector that encodes for the Ins gene
AAV1-ohINS	AAV1 vector that encodes for the human optimized Ins gene
AAV1-Ins+Gck	Solution that contains viral vectors AAV1-Ins and AAV1-Gck
AAV1-hIns+rGck	Solution that contains viral vectors AAV1-hIns and AAV1-rGck
AAV1-null	AAV1 vector that doesn't encode for any protein
AAV1-ohIns+ohGck	Solution that contains viral vectors AAV1-ohIns and AAV1-ohGck
Ad	Adenovirus
AGE	Advanced glycation end-product
ALT	Alanine transaminase
APC	Antigen presenting cells
APECED	Autoimmune polyendocrine syndrome type 1
AST	Aspartate transaminase
ATP	Adenosine triphosphate
BB	Bio breeding
Btc	Betacellulin
bw	Body weight
Ca^{2+}	Calcium
CBATEG	Centre de Biotecnologia Animal i Teràpia Gènica
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
cDNA	Complementary DNA
ClK	Potassium chloride

CMV	Citomegalovirus promoter
CMV-rGck	Rat Gck gene under the control of the CMV promoter
CMV-ohGck	Optimized human Gck gene under the control of the CMV promoter
CMV-hIns	Human Ins gene under the control of the CMV promoter
CMV-ohINS	Optimized human insulin gene under the control of the CMV promoter
Con	Control
CsCl	Cesium chloride
CSII	Continuous subcutaneous insulin infusion system
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CTP	Cytidine triphosphate
Db	Diabetic
DCCT	Diabetes Control and Complications Trial
dl	deciliter
DNA	Deoxyribonucleic acid
ssDNA	single strand Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESRD	End-stage renal disease
FFA	Free fatty acids
g	Grams or graviton
GAD65	Glutamic acid decarboxilase 65
Gck	Glucokinase
GFP	Green fluorescent protein
GH	Growth hormone
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GLUT5	Glucose transporter 5
GLUT7	Glucose transporter 7
Gly	Glycine
GTP	Guanosine triphosphate
GWA	Genome-wide association
h	Hour
H	Healthy
HbA1c	Glycated hemoglobin
HCl	Hydrogen chloride
H&E	Hematoxylin and eosin

HEK293	Human Embryonic Kidney 293 cells
hGck	Human glucokinase
hINS	Human insulin
HKI	Hexokinase 1
HKII	Hexokinase 2
H_2O	Water
IAAs	Insulin autioantibodies
I-A2	Insulinoma-associated antigen 2
IA2β	Insulinoma-associated antigen 2β
ICA69	Islet cell autoantigen 69
IFN-α	Interferon-α
Ins	Insulin
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IPF-1	Insulin promoter factor
ITRs	Inverted terminal repeats
HLA	Human leukocyte antigen
HNF-1α	Hepatocyte nuclear factor 1□
HSV	Herpex Simplex Virus
Kb	Kilobase
kg	Kilogram
KCl	Potassium chloride
K_2CO_3	Potassium carbonate
KDa	Kilodalton
KIU	Kilo international units
km	Kilometer
l	Liter
IDDM1	Insulin-Dependent Diabetes Mellitus 1
IDDM2	Insulin-Dependent Diabetes Mellitus 1
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-10	Interleukin 10
IU	International units
LB	Luria Broth
LV	Lentivirus
M	Molar
MafA	Musculoaponeurotic fibrosarcoma oncogene homolog A

MCS	Multi cloning site
Mg^{2+}	Magnesium
mg	Milligram
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
min	Minutes
mg	Milligram
ml	Milliliter
MLC	Myosin light chain
mM	Millimolar
mmol	Millimol
MOPS	3-(N-morpholino)propanesulfonic acid
MODY	Maturity onset diabetes of the young
mRNA	messenger RNA
n	number of animals, samples or determinations
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
ng	Nanogram
Ngn3	Neurogenin-3
nm	Nanometer
NOD	Non-obese diabetic
NPH	Neutral Protamine Hagedorn
OGTT	Oral glucose tolerance
ohGck	Optimized human Glucokinase
ohINS	Optimized human insulin
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pdx-1	Pancreatic duodenal homeobox-1
PEG	Polyethylene glycol
PEPCK	Phosphoenolpyruvate carboxykinase
PKC	Protein kinase C
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
qPCR	Quantitative real-time polymerase chain reaction
RB	Roller bottle
RBCs	Red blood cells
rGck	Rat Glucokinase

RIA	Radioimmunoassay
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RV	Retrovirus
ScAAV	Self-complementary AAV
SD	Standard deviation
SDS	Sodium dodecyl sulfate
S.E.M	Standard error of the mean
SER-CBATEG	Servei d'Estabulari de Ratolins-Centre de Biotecnologia Animal i Teràpia Gènica
STZ	Streptozotocin
T1D	Type 1 diabetes
TAE	Tris base, acetic acid and EDTA
TBS	Tris-buffered saline
T_{reg}	Regulatory T cells
Tris	Tris(hydroxymethyl)aminomethane
TTP	Deoxythymidine triphosphate
U	Unit
UAB	Universitat Autonoma de Barcelona
mU	Milliunit
µg	Microgram
J	Microjoules
µl	Microliter
µm	Micrometer
V	Volts
vg	Viral genomes
v/v	Volume/Volume
w/v	Weight/Volume
ZnT8	Zinc transporter 8

I. PRESENTATION

Type 1 diabetes patients need insulin replacement therapy to survive, but glycemia is not always regulated in a physiologic manner. Chronic hyperglycemia leads to the development of severe secondary complications that are associated with significant morbidity and mortality. The development of secondary complications can be delayed by tight control of glycemia, however, this is difficult to achieve with exogenous insulin administration because of the associated risk of severe hypoglycemic episodes. Thus, precise regulation of glucose homeostasis is a major challenge in diabetes management.

Genetic engineering of skeletal muscle to counteract hyperglycemia is an attractive strategy to correct diabetes. Skeletal muscle is responsible for the disposal of most (70%) of the circulating glucose after a meal. However, during diabetes, skeletal muscle is unable to take up glucose due to the absence of insulin, a major contributor to the development of hyperglycemia. In addition, skeletal muscle is stable, easily manipulated and is able to secrete proteins into the circulation as well as being transducible by a number of therapeutic vectors.

We previously demonstrated in our laboratory that it is possible to generate a “glucose sensor” in the skeletal muscle through co-expression of glucokinase (Gck) and constitutively basal levels of insulin (Ins). In such a system, glucose flux into skeletal muscle is regulated by circulating glucose levels, allowing increased glucose uptake whenever hyperglycemia is present, but avoiding hpyoglycemia. To test this hypothesis, two adeno-associated viral vectors of serotype 1 (AAV1) expressing Ins and Gck were delivered intramuscularly to diabetic mice, showing correction of the disease.

AAV vectors are the vector of choice for many *in vivo* gene therapy approaches due to their excellent safety and efficacy profile. Pre-clinical studies have shown that AAV vector-mediated gene transfer results in long-term gene expression in small and large animal models of disease. Recently, some of this preclinical data have been translated into humans

with encouraging results. However, for the vast majority of successful proof-of-concept studies in mice, scale-up and long-term efficacy in large animal models or humans has been problematic or disappointing. Scale-up has yet to be demonstrated in large animal models of diabetes with either gene or cell therapy approaches.

In this study, a one-time intramuscular administration of adeno-associated viral vectors of serotype 1 (AAV1) encoding for Gck and Ins in diabetic dogs resulted in normalization of fasting glycemia, normalized disposal of glucose after oral challenge, and no episodes of hypoglycemia during exercise for >4 years after gene transfer. This was associated with recovery of body weight, normal glycosylated plasma proteins levels, and long-term survival without secondary complications. Conversely, exogenous insulin or gene transfer for Ins or Gck alone failed to achieve complete correction of diabetes, indicating that the synergistic action of Ins and Gck are needed for full therapeutic effect.

This demonstration of long-term correction of diabetic hyperglycemia has provided the first proof-of-concept in a large animal model for a gene transfer approach to treat diabetes and lays the foundations for the future translation of this approach to the clinic.

II. INTRODUCTION

1. CONTROL OF GLUCOSE HOMEOSTASIS

Glucose is the main source of energy used by cells, where glucose is oxidized in a catabolic process called cellular respiration, and the energy released is used to synthesize ATP. The energy stored in form of ATP can then be used to drive processes like biosynthesis, transportation of molecules across cell membranes or locomotion (Mathews et al., 2012). Cellular respiration occurs in both eukaryotic and prokaryotic cells and has three main pathways: glycolysis, the citric acid cycle, and electron transport (Mathews et al., 2012).

Alterations in blood glucose levels, like hyperglycemia or hypoglycemia, may have serious physiological consequences and are associated with several disease states, such as diabetes and metabolic syndrome. To maintain homeostasis, blood glucose levels are mainly regulated by the coordinated secretion of insulin and glucagon from the pancreas. Insulin is the only glucose-lowering hormone but there are many physiological antagonists, in addition to glucagon, such as adrenaline and noradrenaline, cortisol, thyroid hormones, gastrointestinal hormones, growth hormone or testosterone. These hormones activate and inhibit different key genes in the metabolism that control glucose production and utilization in different tissues (Mathews et al., 2012).

After a meal, when blood glucose levels increase, there are three processes that synergistically act to guarantee glucose homeostasis. Firstly, glucose stimulates insulin production and release by pancreatic β-cells. Secondly, insulin increases glucose uptake by peripheral tissues such as muscle and adipose tissue. Lastly, high glucose concentration and insulin inhibit glucagon secretion, and this, in turn, leads to a reduction in hepatic glucose production. In fasted conditions, glucagon, whose secretion is elevated, acts with other hormonal factors to increase gluconeogenesis and glycogenolysis and maintain normoglycemia. Thus, any alteration that affects β-cells, the liver, skeletal muscle or adipose

tissue functionality may disrupt glucose homeostasis, causing the development of glucose intolerance or even diabetes mellitus (DeFronzo et al., 1981).

The first step of glucose utilization in the cell is its transport through the cell membrane. This process is done by protein glucose transporters (GLUT1 to GLUT5 and GLUT7) that are structurally related (Mueckler, 1994; Wright et al., 1991). Once inside the cell, hexokinases will phosphorylate the glucose to glucose 6-phosphate that will be stored in form of glycogen or will be metabolized by glycolysis or by the pentose phosphate pathway.

1.1. The role of skeletal muscle in glucose homeostasis

Skeletal muscle constitutes about 40% of body mass and is the major site of glucose disposal after a meal, taking up to 60-70% of circulating glucose in an insulin-dependent-manner, making it an important tissue in glucose homeostasis (DeFronzo et al., 1981).

Skeletal muscle glucose uptake occurs through glucose transporters 1 and 4 (GLUT1 and GLUT4, respectively). Low levels of GLUT1 are expressed in skeletal muscle and are responsible of basal glucose uptake independently of insulin to guarantee minimum carbohydrate availability (Mueckler, 1994; Rea et al., 1997; Wright et al., 1991). In contrast to GLUT1, muscle cells express high levels of the GLUT4 transporter. In basal conditions, this transporter is localized in intracellular vesicles and after a meal, and in response to insulin, the vesicles are translocated to the membrane, increasing glucose transport into the cell (Furtado et al., 2002). This translocation appears to be the key mechanism by which insulin stimulates muscle glucose uptake (Rea et al., 1997).

Once inside the cell, glucose is irreversibly phosphorylated to glucose-6-phosphate by hexokinases. Hexokinase I and II (HKI and HKII) are the predominant isoenzymes in skeletal muscle. HKI is the responsible of the basal glucose phosphorylation and, together with

GLUT1, control non-insulin dependent glucose uptake. HKII, together with GLUT4, control insulin-dependent glucose uptake. Insulin induces translocation of GLUT4 to the cell membrane and increases HKII expression (Figure 1). On the other hand, HKII enzymatic activity and transcription are inhibited when insulin levels are low, or when insulin signalling is altered. It can also be allosterically inhibited by glucose-6-phosphate, the product of its own reaction. This inhibition is a mechanism to control glucose utilization in muscle and other peripheral tissues.

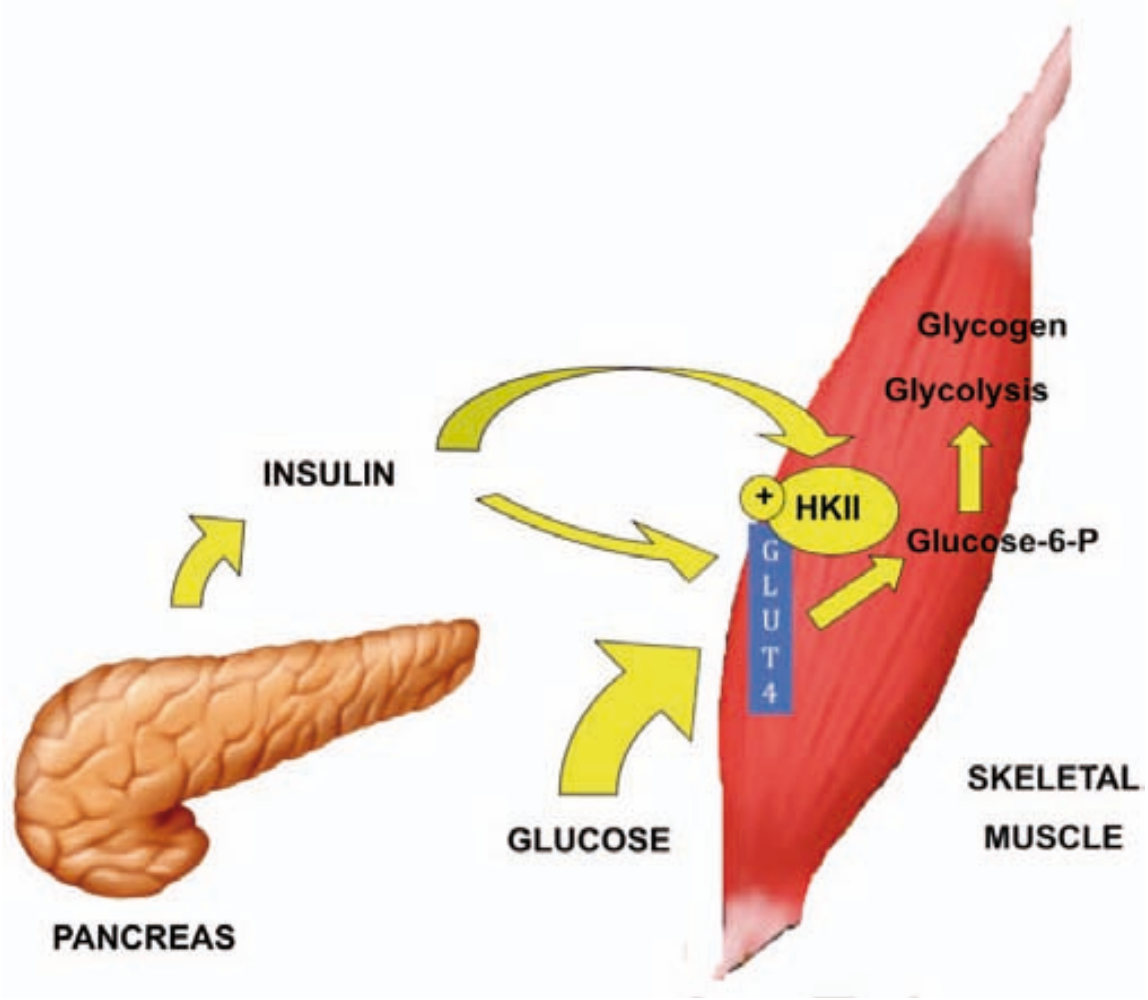

Figure 1. Role of skeletal muscle in glucose homeostasis. In fed conditions, elevated glucose levels lead to insulin release by the pancreas. Insulin in turn induces translocation of glucose transporter GLUT4 to the cell membrane and increases HKII expression, which together increase glucose uptake and utilization by the skeletal muscle. Once inside the cell, glucose is phosphorylated to glucose-6-phosphate by HKII and processed further for either storage, in the form of glycogen, or metabolised, mainly by glycolysis.

1.2. Skeletal muscle structure

There are three distinct types of muscle: skeletal, cardiac and smooth muscle, all of which have both similar and distinct features. Skeletal muscle, also known as striated muscle, is named as such because it is transversely striped or striated due to the arrangement of muscle fibers. A muscle fiber, also known as a myofiber, is a single multinucleated or syncitial cell that results from the fusion of many hundreds of myoblasts. Thus, a muscle fiber is a large cell compared to other types of cells, being 10-60 microns in diameter and several centimetres long. Individual muscle fibers are surrounded by a connective tissue called endomysium (Figure 2). Around 10 to 100 muscle fibers form fascicles, or bundles, which are themselves surrounded by another connective tissue layer called the perimysium. Finally, the skeletal muscle is formed by groups of fascicles that are surrounded also by another connective tissue layer called the epimysium. In addition to muscle fibres, skeletal muscle is also composed of numerous blood vessels and nerves. Moreover, invaginations of the muscle plasma membrane, called the sarcolemma in muscle, form the transverse tubule system perpendicular to the axis of the fiber and facilitate calcium release and contraction. Similarly, the ends of muscles converge in dense connective tissue structures, the tendons and aponeuroses that mediate attachment of muscles to the periosteum of bones or to the connective tissue of other muscles (Figure 2).

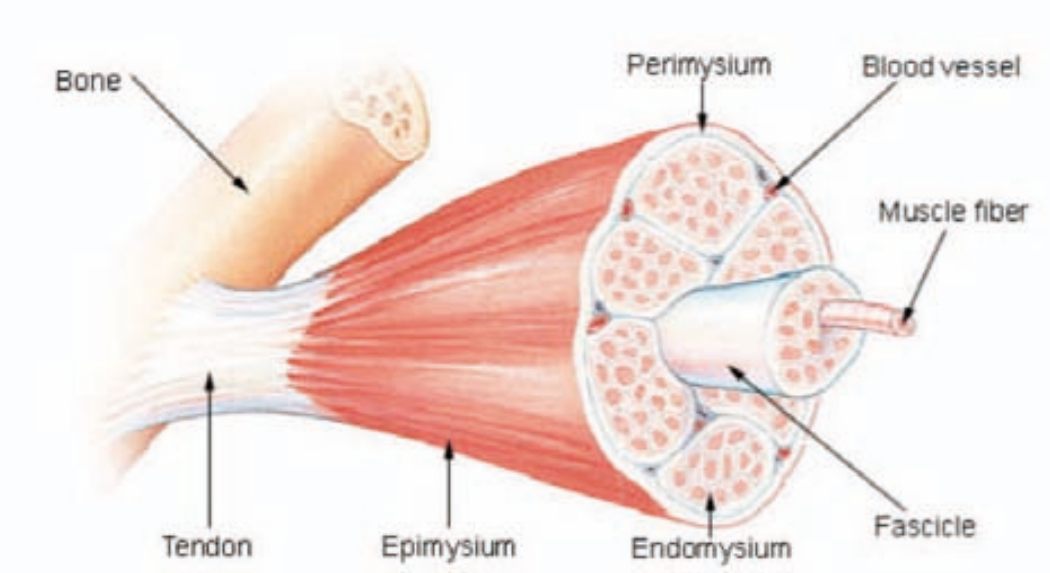

Figure 2. Skeletal muscle structure. Adapted from National Cancer Institute.

The principal cytoplasmic proteins in a muscle fiber are myosin and actin that are arranged in a repeating unit called the sarcomere. The interaction of myosin and actin is responsible for muscle contraction and thus generates movement.

Whole muscle metabolism is dictated by the net composition of the individual fibre types that comprise the muscle and this may change under certain diet, exercise and disease conditions (Mann et al., 2010). Skeletal muscle can be divided into two broad categories based on the type of mysosin they express and the associated metabolic properties. Type I muscle fibers are also called slow fibers and appear red due to the presence of the oxygen binding protein myoglobin and because they are highly vascularized. These fibers have a high content of mitochondria, are suited for endurance and are slow to fatigue because they use oxidative metabolism to generate ATP, which they split at a slow rate, hence, they have a slow contraction velocity (Table 1). Type II fibers are also called fast fibers and can be subdivided in three groups, type IIa and type IIx or type IIb (depending upon the species). Type II fibers are white due to the absence of myoglobin, because they are less vascularised and have less mitochondria. Consequently, they rely on glycolytic enzymes. These fibers are efficient for short bursts of speed and power and use both

oxidative metabolism and anaerobic metabolism. These fibers are quicker to fatigue and they are rich in glycogen and glycolytic enzymes. They have a high capacity for generating ATP by oxidation, splitting ATP at a very rapid rate and, hence, they have a high contraction velocity but fatigue quickly (Table 1).

Table 1. Types and properties of skeletal muscle fibers.

	Type I fibers	Type II a fibers	Type II x fibers	Type II b fibers
Contraction time	Slow	Moderately Fast	Fast	Very fast
Resistance to fatigue	High	Fairly high	Intermediate	Low
Activity used for	Aerobic	Long-term anaerobic	Short-term anaerobic	Short-term anaerobic
Maximum duration of use	Hours	<30 minutes	<5 minutes	<1 minute
Power produced	Low	Medium	High	Very high
Mitochondrial density	High	High	Medium	Low
Capillary density	High	Intermediate	Low	Low
Oxidative capacity	High	High	Intermediate	Low
Glycolytic capacity	Low	High	High	High
Major storage fuel	Triglycerides	Creatine phosphate, glycogen	Creatine phosphate, glycogen	Creatine phosphate, glycogen

Adapted from Mann et al., 2010.

Individual muscles are usually a mixture of all types of muscle fibers (type I, type IIa and type IIb or IIx), but their proportions vary depending on the action of that muscle. For example, deep lying postural muscles are often slow type I.

Exercise is one of the key stimuli able to modify skeletal muscle metabolism and plasticity, for example by changing fuel use and by inducing fibre-type transitions. Firstly, exercise increase glucose uptake in an insulin-independent manner. Also, exercise stimulates GLUT4 translocation, so it stimulates insulin-stimulated glucose uptake. Exercise also leads to activation of several signalling pathways in muscle that are common to insulin

signalling that can affect whole-muscle metabolism. The absence of insulin signalling in diabetic muscle has many consequences, such as increased proteolyisis and altered fuel use, that may affect exercise potential (Mann et al., 2010).

2. DIABETES MELLITUS

Diabetes mellitus is the most common metabolic disease in humans characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association, 2011). Chronic hyperglycemia leads to severe microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (stroke, myocardial infarction) complications (American Diabetes Association, 2011).

Nowadays, diabetes has reached epidemic proportions. An estimated 366 million people were diagnosed to have diabetes in 2011, with the number increasing to 552 million by 2030 (International Diabetes Federation, 2011). However, 80% of people with diabetes live in low- and middle-income countries, and the greatest increases in numbers of people with diabetes over the next 20 years will occur in these demographic groups (International Diabetes Federation, 2011). Diabetes is a major cause of mortality and morbidity. In 2011, diabetes caused 4.6 million deaths in people between 20-79 years of age, accounting for 8.2% of global mortality of people in this age group. Also, diabetes imposes a large economic burden on the individual, national healthcare systems and countries. In 2011, diabetes accounted for 11% of the total healthcare expenditures in the world (International Diabetes Federation, 2011).Thus, diabetes is undoubtedly one of the most challenging health problems in the 21st century.

The vast majority of diabetic patients fall into two broad etiopathogenic categories: type 1 and type 2 diabetes. Type 1 diabetes results from an absolute deficiency of insulin secretion as the consequence of the autoimmune destruction of the pancreatic β-cells. Type 2 diabetes affects approximately 90% of diabetic patients (Zimmet et al., 2001) and is characterized by abnormal insulin secretion, due to an impairment of the β-cell function and a reduction of the β-cell mass, associated with varying degrees of insulin resistance (Butler et

al., 2003; Marchetti et al., 2008). Type 2 diabetes is the result of a complex series of interactions between one or several genetic traits that cause the β-cell dysfuntion and mass reduction, and environmental factors, especially diet and the lack of physical exercise (American Diabetes Association, 1997). As a consequence, type 2 diabetes is strongly associated with obesity, whose worldwide rates are similar to those of diabetes (Kahn et al., 2000; Zimmet et al., 2001). It affects mostly adult individuals, but there are growing numbers of children being diagnosed with the disease due to increases in obesity rates in childhood. Type 2 diabetes is not characterised, at least in the initial stages, by a lack of insulin, but rather by an inability of the hormone to act efficiently on its target tissues such as muscle, liver and adipose tissue (Zimmet et al., 2001). In insulin-resistant states, pancreatic islets usually respond by increasing insulin secretion to maintain normoglycemia, and consequently there is a period of normal or near-normal glycaemia (Kasuga, 2006; Prentki et al., 2006). In these insulin-resistant states, patients show mild hyperglycaemia, hyperinsulinemia and an increase in circulating free fatty acids (FFA). Finally, in susceptible subjects, the pancreas fails to secrete sufficient insulin, due to a β−cell dysfunction and β−cell mass reduction, and diabetes ensues (Prentki et al., 2006; Marchetti et al., 2008). Initially, treatment for insulin resistance is directed towards improving tissue insulin sensitivity. This often involves lifestyle intervention, with modest exercise and weight loss, which clearly reduces the risk of progression to overt diabetes and impaired glucose tolerance. Also, it is very common in the initial stages of the disease to treat patients with oral hypoglycaemic agents. However, in addition to oral hypoglycaemic agents, treatment at later stage of the disease requires exogenous insulin administration to be able to control hyperglycaemia (Stumvoll et al., 2005). Thus, type 1 and type 2 diabetes, ultimately result from an inadequate mass of functional β-cells.

In addition to type 1 and type 2 diabetes, the current classification of the disease includes other several less prevalent disorders such as the gestational diabetes, defined as

any degree of glucose intolerance with onset or first recognition during pregnancy; and diabetes due to an heterogeneous group of monogenic defects in β−cell function, also known as maturity onset diabetes of the young (Zhou et al., 2010; Thanabalasingham et al., 2011). MODYs are estimated to be the underlying cause of diabetes in 1-2% of patients and are typically diagnosed before 25 years old (Thanabalasingham et al., 2011). MODYs are characterized by impaired insulin production or release, with minimal or no defects in insulin action (American Diabetes Association, 2011). Mutations in the genes encoding the enzyme glucokinase (Gck), the nuclear transcription factors hepatocyte nuclear factor 1□ (HNF-1α) and hepatocyte nuclear factor 4□ (HNF-4α) are the most common causes of MODY. Mutations in 10 different loci on different chromosomes have been identified to date. The less common forms include mutations in other transcription factors, including HNF-1β, insulin promoter factor (IPF-1) and NueroD1 (Thanabalasingham et al., 2011).

2.1. Introduction to type 1 diabetes mellitus

Type 1 diabetes is a condition in which pancreatic β-cell destruction leads to an absolute insulin deficiency. Approximately 5 to 10% of diabetic patients are classified as type 1 diabetics (Daneman, 2006). Two forms are identified: type 1A results from a cell-mediated autoimmune attack against β-cells, whereas type 1B is far less frequent, has no known cause, and occurs mostly in individuals of Asian or African descent, who have varying degrees of insulin deficiency between sporadic episodes of ketoacidosis (Daneman, 2006).

Type 1A diabetes generally develops in young subjects, usually before 30 years old, and is characterized by an autoimmune destruction of insulin-producing β−cells of the islets of Langerhans (American Diabetes Association, 1997; Daneman, 2006). There is a persistent and targeted destruction of the β−cells, that may go undetected for many years, and the first clinical symptoms only become apparent after a majority (around 80%) of the

beta-cells have been destroyed or rendered dysfunctional, making the individual dependent on insulin for survival (Knip et al., 2005; Lowe, 1998; van Belle et al., 2011). In the absence of insulin there is a reduction in the uptake and utilization of glucose by insulin-sensitive peripheral tissues, such as muscle and adipose tissue, leading to hyperglycemia (American Diabetes Association, 1997). In addition, glucagon levels are high, which increases the hepatic production of glucose through the glycogenolytic and gluconeogenic pathways, contributing to hyperglycaemia. On the other hand, the lack of endogenous insulin causes a significant degradation of adipose tissue triglycerides and the fatty acids that are released and converted into ketone bodies in the liver that may lead to ketoacidosis if they accumulate. Therefore, the lack of insulin treatment gives rise to hyperglycemia and ketoacidosis, which in turn can lead to a comatose state and eventually the death of the patient if insulin is not administered. Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, polyphagia, and blurred vision. Despite exogenous insulin treatment, sometimes cronic hyperglycemia persists leading to the development of long-term secondary complications (American Diabetes Association, 1997).

2.2. Origin and pathogenesis of type 1 diabetes mellitus

The clinical presentation of type 1 diabetes is preceded by an asymptomatic period of highly variable duration. Aggressive-cell destruction may lead to disease manifestation within a few months in infants and young children, whereas in other individuals, the process may continue for years (in some cases, even for more than 10 years) before the eventual presentation of overt disease (Knip et al., 2005).

Type 1 diabetes develops as a consequence of a combination of genetic predisposition, unknown environmental factors and stochastic events (Bluestone et al., 2010). The precise immunologic, genetic and physiologic events that control disease initiation and progression continue to be investigated, but data from Bio Breeding (BB) rat

and non-obese diabetic (NOD) mouse models, together with analyses of biopsy or autopsy samples from diabetic patients have allowed to develop a more complete model about the key immune events in the development of type 1 diabetes (Figure 3) (van Belle et al., 2011).

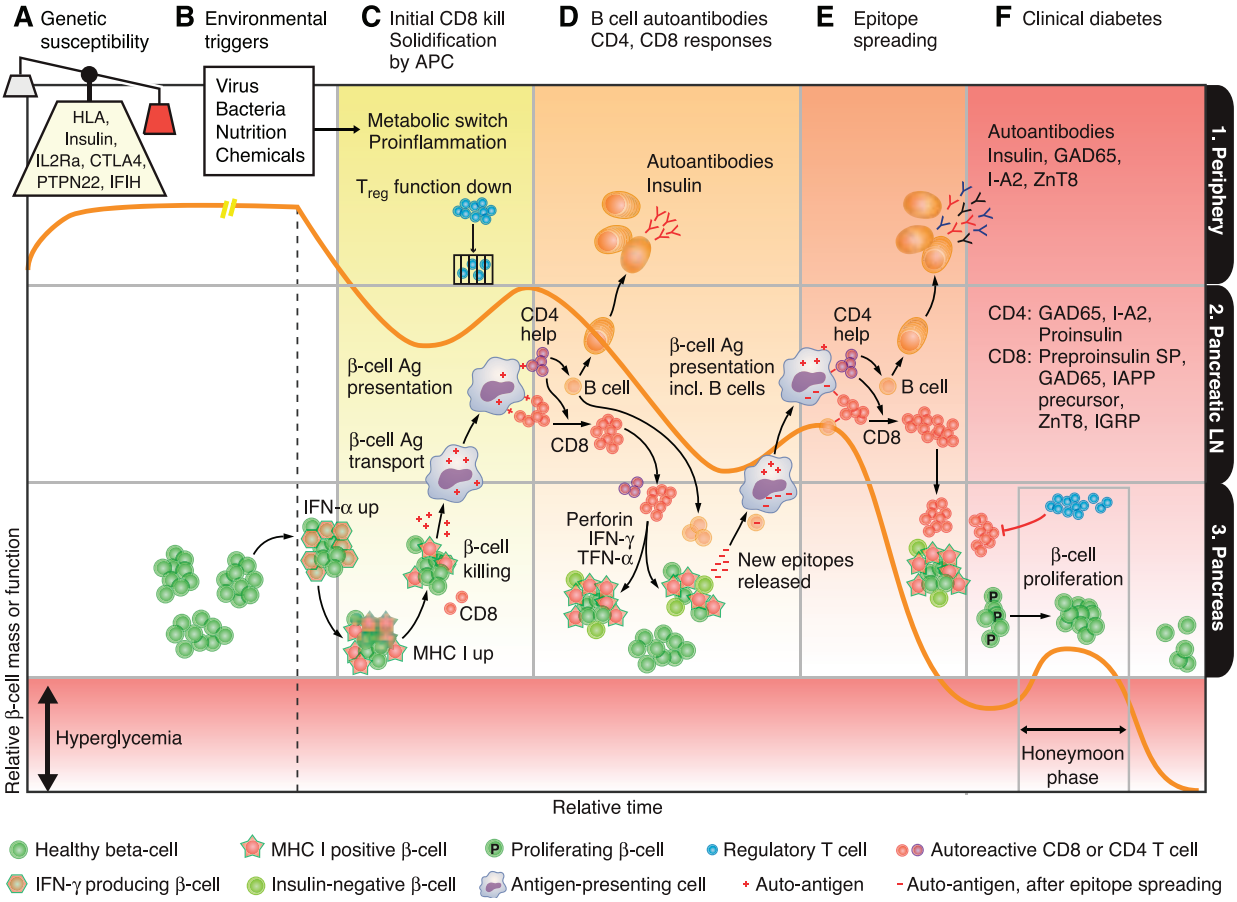

Figure 3. Timeline model for development of type 1 diabetes. This figure represents β-cell mass and/or function (represented by the orange line) as well as the different immunological phases (columns with alphabetized tabs on top) that occur in the relevant anatomical sites (rows with numerical tabs on the right). Once the orange line of ß-cell function falls into the red zone, the individual is clinically diagnosed with type 1 diabetes. Adapted from van Belle et al., 2011.

Islet autoimmunity initiates due to the breakdown in immune regulation in genetically susceptible individuals that encounter certain environmental agents (Figure 3). One theory of the initiation of the islet autoimmunity is the molecular mimicry, that results from cross-reactivity of viral or di□,□*1□□&,□□□&+□/ □ □□-cell antigens that drive deleterious immune responsiveness and □1+□ &□,□&□□□-cell antigen expression □ JR□□□O<G□□□□□□. It has been described that the initiating events of pancreas infiltration include infiltration by dendritic cells,

macrophages and natural killer cells together with autoreactive B-cells (Bluestone et al., 2010). After this, in the pancreatic draining lymph nodes, islet antigens are presented by antigen presenting cells (APC) to $CD4^+$ T cells that in turn activate cytotoxic $CD8^+$ T cells (Figure 3) (Bluestone et al., 2010; van Belle et al., 2011). It has been described that in the pancreas, β-cells upregulate interferon-α (IFN-α) and subsequently MHC class I that exposes β-cells to the attack of autoreactive $CD8^+$ T cells (Figure 3) (van Belle et al., 2011). These activated $CD8^+$ T cells damage the islets, leading to self-antigen release (epitope spreading) and presentation of these new antigens to the immune system that confers new specificities to $CD4^+$ T cells, leading to a newly activated $CD8^+$ T cells. This provokes more β-cell destruction and the cycle begins again (van Belle et al., 2011) (Figure 3). In the case of B-cells, their precise role is less clear, although there is evidence from studies in the NOD mouse model that antibodies and B-lymphocytes contribute to pathogenesis due to autoantibody production and because they act as a very efficient APC (Bluestone et al., 2010; Miao et al., 2007). Described autoantibodies include insulin autioantibodies (IAAs) or antibodies directed against insulinoma-associated antigen 2 (I-A2 or ICA512), insulinoma-associated antigen 2β (IA2β), glutamic acid decarboxilase 65 (GAD65), islet cell autoantigen (ICA69), trisialo-ganglioside GT3, ganglioside GM2-1 and zinc transporter 8 (ZnT8) (Knip et al., 2005; Miao et al., 2007; van Belle et al., 2011). It has been described in several studies that regulatory T cells (T_{reg}) number and function are altered during disease progression, suggesting that progression of the autoimmune destruction may be a consequence of the imbalance of T_{reg} and effector T cells (Bluestone et al., 2010). At the end stage of the disease development, when clinical presentation of diabetes occurs, somewhere between 60 and 90% of the β-cells have been destroyed or are dysfunctional (van Belle et al., 2011).

Despite ongoing □-cell destruction, the honeymoon phase is a phenomenon that occurs in up to 60% of type 1 diabetes patients immediately after diagnosis and initiation of insulin treatment and is characterized by a reduced insulin requirement while good metabolic

control is maintained. This is usually an incomplete remission period because, except in a few cases, a small insulin dose is still required. The duration of this period ranges between 1 month and 13 years, but usually lasts from 3 to 6 months. The mechanisms governing this improved β-cell function are poorly understood, but it is thought that the constant hyperglycemic stimulus that exhausts the remaining β-cells can be temporarily relieved by the insulin treatment and the remaining cells can replenish their insulin content (Abdul-Rasoul et al., 2006; van Belle et al., 2011).

2.3. Genetics of type 1 diabetes

Genetic predisposition is a major risk factor for type 1 diabetes. For example, in family studies, a concordance rate of 13 and 40% in monozygotic, compared to 5% in dizygotic twins, demonstrates a significant genetic component in disease susceptibility (Hewagama et al., 2009; Knip et al., 2005).

However, autoimmune diabetes is only rarely caused by mutational defects in a single gene. Examples of these monogenic forms include the IPEX syndrome, or the autoimmune polyendocrine syndrome type 1 (APECED) (Daneman, 2006; van Belle et al., 2011).

Early studies indicated that the HLA region on chromosome 6p21 (commonly termed IDDM1, for insulin-dependent diabetes mellitus locus) is a critical susceptibility locus for many human autoimmune diseases, including T1D (van Belle et al., 2011). The HLA region represents the strongest association found with the risk of development of T1D, because is thought to confer about 50% of the genetic usceptibility. Several HLA genes are pivotal as their alleles were found to determine a susceptibility hierarchy ranging from protection (DRB1*1501- DQA1*0102-DQB1*0602) to susceptibility (DR3/4-DQ8) (Daneman, 2006; van Belle et al., 2011). The other two major susceptibility locus, that confer 15% of genetic susceptibility, are the IDDM2, which includes the insulin gene region and the IDDM12, that

includes the cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (Daneman, 2006; van Belle et al., 2011).

Other suscepitility locus include PTPN22 or LYP which encodes a lymphoid protein tyrosine phosphatase and the interleukin (IL)-2 receptor-gene (IL2RA) region (van Belle et al., 2011).

The most recent genome-wide association (GWA) study focusing on T1D found that over 40 loci affect risk of type 1 diabetes. One of the major conclusions from such a comprehensive studies was that autoimmune diseases share many genetic risk factors, suggesting common underlying pathways (van Belle et al., 2011).

2.4. Environmental factors in type 1 diabetes

A series of evidence supports a critical role of exogenous factors in the development of type 1 diabetes, such as 1) the fact that <10% of individuals with HLA-conferred diabetes susceptibility progress to clinical disease; 2) a pairwise concordance of type 1 diabetes between 13-40% among monozygotic twins; 3) a >10-fold difference in the disease incidence among Caucasians living in Europe, a difference in incidence that can hardly be explained by genetic factors; 4) a several-fold increase in the incidence over the last 50 years; and 5) migration studies indicating that the disease incidence has increased in population groups who have moved from a low-incidence to a high-incidence region (Hewagama et al., 2009; Knip et al., 2005).

The main environmental triggers known today are first of all viral infections by Enterovirus (more specifically coxsackieviruses), rotaviruses and congenital rubella (Buschard, 2011; van Belle et al., 2011). Other environmental factors are the bacterial composition of the intestine,

enviromental toxins like nitrosamine and the consumption of wheat proteins, more specifically, gluten (van Belle et al., 2011).

The so-called ‘‘hygiene hypothesis’’ has also been proposed which postulates that our environment for young infants is too clean, with reduced exposure to parasites, leading to a deficiency in immuneregulation (Bluestone et al., 2010; Devendra et al., 2004).

2.5. Diabetes-associated long-term complications

Type 1 diabetes is diagnosed when □-cell destruction is almost complete and patients need insulin replacement therapy to survive. However, even with insulin replacement therapy, glycemia is not always properly regulated, and chronic hyperglycemia leads to severe microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (stroke, myocardial infarction) complications (American Diabetes Association, 2011). Mortality rate is up to 5 times higher in diabetic than in the non-diabetic population (Marchetti et al., 2008). These devastating complications can be prevented by normalization of blood glucose levels, which is sometimes very difficult to achieve.

There are four main hypotheses about how hyperglycaemia causes diabetic complications: 1) increased polyol pathway flux; 2) increased advanced glycation end-product (AGE) formation; 3) activation of protein kinase C (PKC) isoforms; and 4) increased hexosamine pathway flux. There is a unifying hypothesis linking these four mechanisms which is that each of the four different pathogenic mechanisms reflects a single hyperglycaemia-induced process: overproduction of superoxide by the mitochondrial electron-transport chain (Brownlee, 2001).

2.5.1. Microvascular complications

Diabetic nephropathy is the most common cause of renal failure in the developed world. The proportion of individuals who progress to end-stage renal disease (ESRD) used to be estimated as 30–40% (Daneman et al., 2006). However, more recent data suggest that this proportion is decreasing, probably as a result of intensive efforts to control both glycaemia and hypertension (Finne et al., 2005). There is also a close association between diabetic retinopathy and diabetic nephropathy (Daneman, 2006).

Diabetic retinopathy is the most common cause of acquired blindness in the western world. The prevalence rate of proliferative retinopathy is about 20–25% in type 1 diabetes (Daneman, 2006). It progresses from early non-proliferative retinopathy, characterized by alterations in the retinal vasculature membrane, to proliferative retinopathy, associated with overt neovascularization, vitreous haemorrhage and risk of retinal detachment and visual loss (Cai et al., 2002). Other ocular complications with lower prevalence are glaucoma, cataracts and neovascularization of the iris (Rand et al., 2004).

Another macrovascular complication is the diabetic neuropathy that refers to a complex group of conditions falling into two major categories: focal and generalised. Focal neuropathies include, for example, carpal tunnel syndrome, peroneal nerve and third cranial nerve palsies, and diabetic amyotrophy (proximal nerve conditions). The most common generalised neuropathy is sensorimotor polyneuropathy, which often first presents as a peripheral neuropathy alone, but may also affect the autonomic system with cardiac dysfunction, gastroparesis, and erectile dysfunction (Daneman, 2006).

Peripheral neuropathy, in conjunction with peripheral vascular disease, can lead to skin ulceration of the lower limbs, poor healing and gangrene, and even amputation in severe cases of so-called “diabetic foot“ (Daneman, 2006).

2.5.2. Macrovascular complications

The macrovascular complications include cardiovascular disease, cerebrovascular accidents, and peripheral vascular disease (Melendez-Ramirez et al., 2010), with cardiovascular disease being the most common cause of death and disability among people with diabetes (International Diabetes Federation, 2011).

The relative risk of cardiovascular disease in type 1 diabetes can be as much as 10-fold greater than that in non-diabetic individuals of the same age. Risk factors for cardiovascular disease in type 1 diabetes include the presence of diabetic nephropathy, but also autonomic neuropathy, dyslipidaemia, hypertension, and perhaps also specific microvascular cardiac disease (Daneman, 2006).

3. DIABETES TREATMENT

3.1. Clinical management of type 1 diabetes

3.1.1. Substitutive therapy with insulin

The reduction of hyperglycemia and maintenance of normoglycemia is the goal of any therapeutic approach to type 1 diabetes and insulin is widely accepted to be the most glucose-lowering agent (Bergenstal, 2004). Nowadays, five types of insulin exist for the treatment of type 1 diabetes:

1) *Rapid-acting insulin* has the fastest peak action, the fastest onset of action and the shortest duration of action. Nowadays, this insulin is used to target postpandrial hyperglycemia due to its rapid onset and peak.

2) *Short-acting or regular insulin* has a slower onset of action, a later peak action and longer duration of action than the rapid-acting insulin, which have been shown to result in better postpandrial control with less hypoglycemic episodes.

3) *Intermediate-acting insulin*. They include the Neutral Protamine Hagedorn (NPH) and the lente insulin (Mori et al., 2008). If either excess zinc (lente insulin) or protein protamine (NPH) is added to short-acting regular insulin or rapid-acting insulin analogue formulations, this slows the rate of insulin's absorption from the subcutaneous tissue into the circulation. They have been traditionally used as basal insulin before the appareance of the long-acting insulin analogues (Bergenstal, 2004).

4) *Prolonged-intermediate-acting insulin.* Ultralente insulin is an extended zinc formulation of lente insulin. Ultralente has an onset and peak similar to that of intermediate-

acting insulin but a bit later or prolonged. Ultralente has a duration that is longer than that of intermediate, but not as long as newer longer acting insulin preparations such as insulin glargine. In most clinical cases it has been used twice a day or in a regimen in which is given once per day combined with NPH.

5) *Long-acting insulin*. Long-acting insulins include insulin glargine and insulin detemir. Insulin glargine is an insulin analog that has a relatively flat insulin curve and duration close to 24 hours, and it is approved for use once daily (Bergenstal, 2004). Insulin detemir is another long-acting insulin analogue that is a competitor of insulin glargine and holds the advantage that probably has less intrapatient variability. Insulin glargine and insulin detemir differ in terms of potency and duration of action because detemir should be given twice daily in the large majority of people with type 1 diabetes (Rossetti et al., 2008). A new type of insulin is in clinical development, an ultra long-acting insulin called insulin degludec. Pharmacokinetic data have shown that insulin degludec has a flat, stable profile at steady state and a terminal half-life of more than 25 hours, which is twice that of insulin glargine, and duration of action greater than 40 hours. In a recent phase III trial, insulin degludec have provided similar glycemic control to insulin glargine in a basal-bolus regimen with rapid-acting insulin aspart as meal-time insulin with a lower rate of nocturnal hypoglycaemia, suggesting a potential role for insulin degludec in helping patients with type 1 diabetes to reach and maintain tight glucose targets.

Type 1 diabetic patients are best managed with an intensive insulin therapy that consists on three or more daily injections with a basal-bolus insulin regimen (Bergenstal, 2004; Daneman, 2006). It was shown in the Diabetes Control and Complications Trial (DCCT) that patients who followed an intensive insulin therapy had a tighter glycemic control (measured by haemoglobin A1c (HbA1c) concentrations) than those who followed conventional insulin treatment (1-2 injections per day) (Diabetes Control and Complications Trial, 1993). This improved glycemic control, maintained over the long term, delayed the

onset and progression of long-term secondary complications (Diabetes Control and Complications Trial, 1993). Multiple daily injections routines have traditionally consisted of NPH or ultralente given once or twice daily as the basal insulin, with regular human insulin boluses before meals (Daneman, 2006). With the availability of both fast-acting and long-acting insulin analogues multiple daily injection routines mostly use insulin glargine or detemir as the basal insulin and insulin lispro or insulin aspart as the boluses before meals (Bergenstal, 2004; Daneman, 2006; Rossetti et al., 2008). However, patients undertaking intensive insulin treatment present a higher risk of hypoglycemia, that is the major limiting step to achieve better glycemic control (Daneman, 2006; Diabetes Control and Complications Trial, 1993). Also, it has been described in a more recent study in patients receiving intensive insulin therapy, that the rate of development of secondary complications is much higher than those described in the previous DCCT trial (Diabetes Control and Complications Trial, 1993; Nathan et al., 2009).

In addition, the intensive insulin therapy is not suitable for all diabetic patients, especially the very young or the old, because the risk of hypoglycemia is higher in these population groups. Insulin treatment is especially difficult in a small group of patients that suffer brittle diabetes. Brittle diabetes affects 3/1000 insulin-dependent diabetic patients, mainly young women, and is characterized by a severe instability of glycemic values with frequent and unpredictable episodes of hypoglycemia and/or ketoacidosis which cannot be explained by errors of patients or diabetologists (Bertuzzi et al., 2007; Vantyghem et al., 2006). The quality of life of these patients is dramatically compromised because of the frequency of acute events, hospital recoveries, the precocious appearance of chronic complications and shortened life expectancy (Vantyghem et al., 2006). Pancreas or islet transplantation represents an effective therapeutic option for these patients, taking in account the limitations of these treatments. However, the introduction of insulin analogs and the use of subcutaneous insulin pumps have significantly increased the possibility of treating most of

these cases, although there is a minority of patients resistant to the therapy (Bertuzzi et al., 2007).

Other typical complications of insulin therapy are the Somogy effect and the Dawn phenomenon. The Somogy effect is caused by nighttime hypoglycemia, which leads to a rebound hyperglycemia in the early morning hours. It is a result of having extra insulin before bedtime, either from not having a bedtime snack, or from long-acting insulins (Monahan et al., 2007). The Dawn phenomenon is a sudden rise in blood glucose levels in the early morning hours, that unlike the Somogyi effect, it is not the result of antecedent hypoglycemia (Atiea et al., 1992; Schmidt et al., 1981). It is believed to be caused by the natural overnight release of hormones (growth hormones, cortisol, glucagon and epinephrine) that increases insulin resistance and/or by insufficient insulin the night before, incorrect medication dosages or carbohydrate snack consumption at bedtime (Monahan et al., 2007).

Finally, in many underdeveloped countries, especially in less privileged families, access to self-care tools and also to insulin is limited, which may lead to severe handicaps and early death in diabetic children. The most common cause of death in a child with diabetes, from a global perspective, is lack of access to insulin (Beran et al., 2006; International Diabetes Federation, 2011; Gale, 2006). Thus, an increased uniform implementation of therapies known to be effective or the development of more effective therapies is needed.

3.1.2. Insulin pump therapy

Alternatively, it can be used an external pump in a continuous subcutaneous insulin infusion system (CSII) that infuses a rapid acting insulin analogue (Bergenstal, 2004). Compared with conventional multiple daily injection therapy, CSII can provide a small, but clinically important reduction of HbA1c levels, specially in individuals who had severe

hypoglycemia, diminished blood glucose variability and up to threefold reduction in severe hypoglycemic episodes (Bruttomesso et al., 2009; Hanaire et al., 2008; Pickup et al., 2008; White, 2007). Furthermore, CSII benefits include positive effects on quality of life and the possibility to adjust insulin administration according to physical activity or food intake (Bruttomesso et al., 2009; White, 2007). However, the disadvantages of CSII include the possibility of technical problems with the pump, especially failures at night where patients can be deprived of insulin for many hours and develop diabetic ketoacidosis. Other problems include the possibility of infection and inflammation of the infusion site, a body weight increase, the requirement for specially trained personnel and the increased cost of CSII compared to multiple daily injections (Bruttomesso et al., 2009; White, 2007).

3.1.3. Pancreas transplantation

Pancreas transplantation was first described in 1967, but initial pancreas graft and patient survival were very poor (Kelly et al., 1967). Advances in surgical techniques, immunosuppresion, graft preservation techniques, methods of diagnosis, treatment of rejection and management of common transplantations complications have led to significant improvements in graft and patient survival (Gruessner et al., 2002).

There are three main types of pancreas transplantation: 1) simultaneous pancreas-kidney transplant, in which the pancreas and kidney are transplanted from the same deceased donor; 2) pancreas after-kidney transplant; and 3) pancreas transplant alone for the patient with type 1 diabetes who presents frequent hypoglycemia, but adequate kidney function (Larsen, 2004). The operation itself is serious, because one to two people in 10 die within a year of getting a pancreas transplant (Venstrom et al., 2003). It has been demonstrated that, for patients with functioning kidneys, survival rates for pancreas-only transplants are worse than the survival rates of patients who receive a pancreas and a kidney trasnplantation together. So, combined transplant of kidney and pancreas is less likely

to fail (Venstrom et al., 2003). Therefore, in most cases, pancreas transplantation is performed in the setting of type 1 diabetes with ESRD, in patients who will receive, or who already have, a transplanted kidney (Venstrom et al., 2003).

Limitations of pancreas transplantation come mainly for the need for life-long immunosuppression, the side effects of these immunosuppressive drugs and the shortage of pancreas donors (Venstrom et al., 2003).

3.1.4. Islet transplantation

Islet transplantation has been investigated as a treatment for type 1 diabetes in selected patients with inadequate glucose control despite insulin therapy and with a high frequency of severe hypoglycemic episodes. However, the hope that such an approach would result in long-term freedom from the need of exogenous insulin has failed to materialize in practice.

Between 1990 and 1998, only 12% of islet allotransplants resulted in insulin independence for periods of more than one week, and only 8.2% have done so for a periods of more than one year, because the regimen of immunosuppression harmed the transplanted islets (White et al., 2001). To address this problem, the Edmonton protocol described by Shapiro and collaborators in 2000 proposed a new immunosuppressive protocol (Shapiro et al., 2000). Recent data revealed that with this new protocol 72% of all recipients became insulin-independent after transplantation, but more than 50% of those with insulin independence were back on insulin within 2 years (Halban et al., 2010). The median duration of insulin independence was 15 months (Ryan et al., 2005). Despite the return to insulin injections in most of transplanted patients, the concomitant (low grade) insulin secretion from the remaining graft still smoothed glycemic control in many of these patients and reduced the incidence of hypoglycemia. However, progression to complete graft failure occurred within a

few years, due to a continuing assault by autoimmunity and allorejection, as well as toxicity of immunosuppressive drugs (Halban et al., 2010). This limitations indicate that the procedure in its current format is not suitable for all patients with type 1 diabetes (Correa-Giannella et al., 2009).

One key obstacle for further progress of istet transplantation is the need to increase the availability of transplantable islets, eliminating the need for multiple islet donors per recipient. Additionally, the protocol need to be improved by solving problems related to cell loss during the process of islet isolation, improving islet engraftment by reducing graft site apoptosis, allorejection and autoimmunity, and reducing toxic immunosuppression (Correa-Giannella et al., 2009). The development of new immunosuppressive agents and strategies to induce immunological tolerance could help in reducing the toxicity of immunosuppression (Correa-Giannella et al., 2009; Halban et al., 2010).

3.2. Gene therapy

3.2.1. Introduction to gene therapy

Gene therapy can be broadly defined as the introduction of genetic material using a vector, into a cell, tissue or whole organ, with the goal of curing a disease or at least improving the clinical status of a patient (Verma et al., 2005).

Vectors can be administered directly in *vivo* or used *ex vivo* to modify cells prior to transplantation, where the cells are preferably autologous cells derived from an individual patient to minimise immune rejection (Kay, 2011).

A key factor in the success of *in vivo* gene therapy is the development of gene delivery systems that are capable of efficient gene transfer in a broad variety of tissues, without causing any pathogenic effect. Depending on the vector, the therapeutic DNA either integrates into host chromosomal DNA or exists as an episomal vector (Kay, 2011). The vectors that have been developed can roughly be divided in two categories: non-viral and viral vectors.

3.2.2 Recombinant viral vectors

Viruses represent highly evolved natural vectors for the transfer of foreign genetic information into cells. This attribute has led to extensive attempts to engineer recombinant viral vectors for the delivery of therapeutic genes into diseased tissues (Kay et al., 2001).

The viral life cycle can be divided into two temporally distinct phases: infection and replication. Infection results in the introduction of the viral genome into a cell. This lead to an early phase of gene expression characterized by the appearance of viral regulatory products,

followed by a late phase, when structural genes are expressed and assembly of new viral particles occur. The first step to design a viral vector is to identify the viral sequences required for replication, assembly of the viral particles, packaging of the viral genome and delivery of the transgene into the target cells and to remove all the possible pathogenic sequences, leaving only the required sequences for packaging and transgene delivery (Verma et al., 2005). Viral vectors encapsulate a modified genome carrying a therapeutic gene cassette in place of the viral genome. Transduction is defined as an abortive infection that introduces this functional genetic information expressed from the recombinant vectors into the target cell (Figure 5) (Kay et al., 2001).

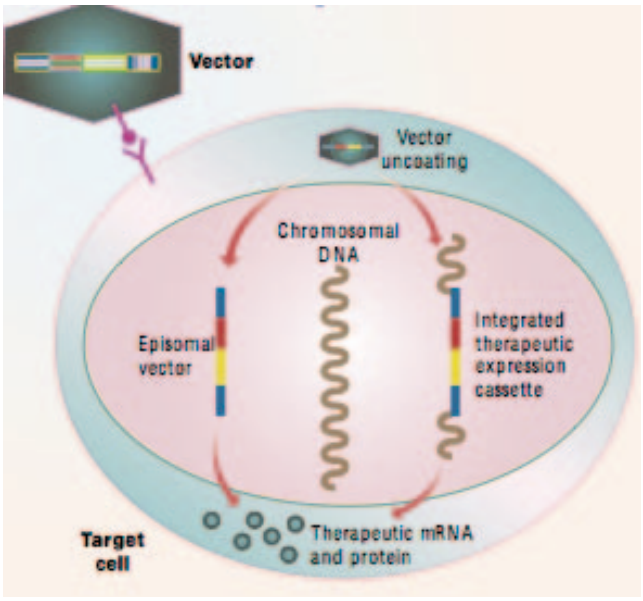

Figure 5. Transduction of a target cell with a viral vector. The vector particle containing the therapeutic gene binds to a cell, generally through a receptor-mediated process and then enters the cell, allowing the genome to enter the nucleus. The vector genome may go through complex processing, but ultimately becomes dsDNA that, depending on the vector, can persist as an episome or become integrated into the host genome. Once in the nucleus, expression of the therapeutic gene proceeds from ubuiquitous or tissue-specific promoters. Adapted from Kay et al., 2001.

For a successful gene therapy, an appropriate amount of the therapeutic gene must be delivered into the target tissue without substantial toxicity. Each viral vector system is characterized by an inherent set of properties that affect its suitability for specific applications (Table 2). For some disorders in which the protein does not require to be expressed in every

single cell of a tissue or organ, long-term expression from a relatively small proportion of cells would be enough (for example in genetic diseased where the missing protein is a circulating protein). In other cases long-term expression from a big percentage of a tissue or organ will be required (for example when the missing protein is intracellular), whereas in other type of pathologies (like for example cancer) high but transient gene expression would be necessary (Kay et al., 2001). Table 2 summarizes the inherent properties of the most commonly used viral vectors in gene therapy over the last years.

One of the potential complications of viral gene therapy include is immunotoxicity that can be due to: 1) acute toxicity of the vector resulting from an innate immune response; 2) an adaptative response, either cellular against the viral capsid and transduced cells, or humoral against the vector that will prevent transduction in pre-immunized individuals or readministration; and/or 3) an immune response against the therapeutic gene product.

Table 2. General characteristics of the most commonly used viral vectors.

Vector type	Advantages	Disadvantages
Adenovirus (Ad)	• High encapsulation capacity (36 kb) • Useful for proof of concept experiments and/or experiments were short-term expression is required. • Production in high titers • They transduce with high eficiency a wide range of tissues. • Retargeting is possible • Remains episomal	• Very immunogenic *in vivo*. • Short-term expression due to the immunogenicity. • Unselective tropism
Adeno-associated virus (AAV)	• Very weak immunogenicity. • Infects quiescent and dividing cells • Remains episomal (mostly) • Production in high titers • Non-pathogenic • Several serotypes with different tropism transduce quite selectively different tissues or a wide range of tissues • High stability in specific cell lines	• Low encapsulation capacity (4.8 kb)
Retrovirus (RV)	• Stable expression in dividing cells. • Retargeting is possible. • High titers are easily obtained.	• Random integration into the host genome, possible risk of insertional mutagenesis. • Infects only dividing cells • Limited encapsulation capacity (8 kb)
Lentivirus (LV)	• Non immunogenic. • Stable expression for long periods of time • Infects quiescent and dividing cells • High titers are easily obtained with an abscence of replicative competent particles in the viral preparations	• Random integration into the host genome, possible risk of insertional mutagenesis. • More studies are needed to assess the lack of pathogenicity
Herpes Simplex Virus (HSV)	• High encapsulation capacity (50 kb) • Retargeting is possible • Remains episomal • Infects quiescent and dividing cells	• Poorly characterized • Transient expression

Adapted from Verma et al., 2005.

3.2.3. Adenoassociated vectors (AAV)

The lack of pathogenicity of the AAVs, their excellent safety profile, the persistence of the virus in a wide variety of tissues, and the many available serotypes have increased AAVs potential as a delivery vehicle for gene therapy applications (Daya et al., 2008). Currently, most strategies for treating human genetic diseases by means of *in vivo* gene therapy exploit AAVs and the most promising clinical results have also been achieved using these vectors (Mingozzi et al., 2011).

3.2.3.1. *Biology of AAV*

AAVs are small non-enveloped viruses with an icosahedral capsid that has a diameter of 20-25nm and a single stranded DNA genome that is approximately 4.7 kb in size (Mingozzi et al., 2011). AAVs are grouped in the familiy *Parvoviridae* in the genus *Dependovirus*. AAV has never been shown to cause any human disease, despite a high seroprevalence rate. To date 14 serotypes and more than 100 variants have been described (Buning et al., 2008), but only a few have been incorporated into vectors and studied in depth (Kay, 2011). AAV serotypes 1 to 6 were isolated as contaminants in laboratory adenovirus stocks with exception of AAV5 (Wu et al., 2006). AAV6 was characterized in 1999 and appears to be a recombinant between AAV1 and AAV2 (Xiao et al., 1999). Later, five new serotypes (AAV7, AAV8, AAV9, AAV10 and AAV11) were isolated from non-human primates (Gao et al., 2002; Mori et al., 2004). All serotypes have in common a similar size and genomic configurations of replication and structural genes. AAV2 is the best characterized and has been the most frequently employed recombinant AAV vector (Mingozzi et al., 2011).

The life-cycle of AAVs has been relatively well described. The different serotypes of AAV utilize a variety of approaches using different receptors and coreceptors for cell entry, and this results in different host ranges (Verma et al., 2005). After binding to its receptor, the

virus enters the cell through receptor-mediated endocytosis and is subsequently transported to the nucleus (Verma et al., 2005). Viral uncoating in the nucleus releases the single-stranded genome that then needs to be converted to a double-stranded form to enable gene expression (Verma et al., 2005). In the presence of a helper virus such as Adeno, Herpes or Vaccinia virus or in response to a variety of cellular stresses, the genome is actively transcribed and replicated (Schlehofer et al., 1986). This step is performed mostly by the cellular DNA and RNA synthesis machineries and requires the presence of AAV proteins encoded by the rep gene (Buning et al., 2008). Viral capsids assemble in the nucleus, the replicated genome is packaged and virions are liberated upon cell lysis (Wistuba et al., 1997). In the absence of adenovirus AAV can establish latency by specifically integration into a 4-kb region on chromosome 19 (Giraud et al., 1994; Kotin et al., 1990; Samulski et al., 1991).

3.2.3.2. *AAV based vectors*

AAV vectors are generated by replacing rep and cap genes in the viral genome by the construct of choice, keeping only the ITRs because they are the *cis* signals required for packaging. As a consequence, AAV vectors are devoid of any viral genes, thus they are not replicative.

To choose the AAV serotype that best fits for one application, it is important to consider both, the serotype efficiency transduction in the target organ and the specificity of transduction for the possible undesired transduction of off-target organs (Buning et al., 2008). For transducing skeletal muscle, AAV1 and AAV7 are the best serotypes, but AAV6, AAV8 and AAV9 also show some capacity (Wu et al., 2006). It has been described that in the skeletal muscle AAV1 vectors allow sustained long-term expression in animal models and humans (Chao et al., 2000; Arruda et al., 2004; Brantly et al., 2009; Chao et al., 2001; Mendell et al., 2009; Mingozzi et al., 2009; Riviere et al., 2006; Xiao et al., 1999).

AAV vectors can transduce dividing and non-dividing cells and most (>99%) proviral AAV genomes remain episomal in the nucleus of the host cell, as concatemers of vector DNA (Kay, 2011). The efficiency of AAV vector transduction is dependent on the efficiency at each step of AAV infection being the inefficient AAV trafficking and second-strand synthesis the rate-limiting factors in AAV gene expression (McCarty et al., 2001). Self-complementary AAV (scAAV) vectors can fold upon themselves, immediately forming transcriptionally competent double-stranded DNA. scAAV vectors bypass the limiting aspects of second-strand synthesis and shorten the lag time before transgene expression resulting in the production of higher levels of transgene products (Nathwani et al., 2007; Wu et al., 2008).

3.2.3.3. *AAV vectors in clinical trials*

Recombinant AAV vectors have gained popularity in clinical trials and in fact the most promising results to date have been achieved using them.

Succesful clinical trials have included targeting the subretinal space for treatment of Leber's congenital amaurosis (Maguire et al., 2009; Maguire et al., 2008), the brain for treatment of Parkinson disease (Christine et al., 2009; Eberling et al., 2008; Kaplitt et al., 2007; Marks et al., 2008; Muramatsu et al., 2010) or the liver for the treatment of hemophilia B (Nathwani et al., 2011).

Skeletal muscle is an attractive target tissue for gene transfer for treating neuromuscular diseases, metabolic diseases and inherited protein diseases, because it has several distinct features: 1) it is easily accessible by non-invasive procedures; 2) it can be transduced by many viral and non-viral systems; 3) it can efficiently secrete proteins into the bloodstream; 4) due to the slow turnover and limited proliferation, expression is stable for many years and risks of insertional mutagenesis are low; 5) gene delivery is not limited by the presence of pre-existing neutralizing antibodies; and 6) vector delivery leads to minimal

systemic biodistribution reducing genotoxicty (Kay, 2011) (Manno et al., 2003; Mingozzi et al., 2011). Successfully studies have been reported like for instance the one for alpha-1 antitrypsin deficiency (Brantly et al., 2009; Brantly et al., 2006; Flotte et al., 2011). Muscular dystrophies are also an attractive target for gene therapy and successful clinical trial has been reported in Limb-Girdle Muscular Dystrophy Type 2D (Mendell et al., 2009).

3.2.4. Gene therapy for type 1 diabetes

Limitations of the current therapies for type 1 diabetes could be potentially circumvented with gene therapy. Nevertheless, in contrast with other human genetic diseases caused by a mutation in a single gene in a defined target tissue, type 1 diabetes is a multi-factorial disease with many stages affecting whole-body metabolism and thus there are many potential target tissues and therapeutic genes (Efrat, 1998; Levine et al., 1999). For this reason, it is necessary to approach the disease with different gene therapy strategies which can be broadly be divided into three categories: 1) Preventive and adjunctive therapy: in which the autoimmune pathogenesis is addressed to avoid the destruction of the remaining β-cells or improve the graft survival after islet cell transplant; 2) β-cell neogenesis and regeneration: in which key genes or factors are introduced in the remaining β-cells, β-cell precursors, in the exocrine pancreas or in extrapancreatic tissues to produce new β-cells; and 3) genetic modification of extra-pancreatic tissues to express insulin and/or increase glucose uptake to reverse diabetic hyperglycemia .

3.2.4.1. *Gene therapy to induce immune tolerance or improve graft survival*

Primary prevention of type 1 diabetes involves identifying patients at risk whereas secondary prevention are measures to prevent further loss of β cells once diabetes has been recently diagnosed or in the honeymoon period (Agardh et al., 2005; Chaillous et al., 2000; Chong et al., 2006; Ergun-Longmire et al., 2004; Herold et al., 2002; Keymeulen et al., 2005; Keymeulen et al., 2010; Kodama et al., 2003; Suri et al., 2006; Zhao et al., 2012).

Immunomodulatory gene therapy to induce tolerance or hyporesponsiveness to islet autoantigens may be useful to prevent or halt the autoimmune destruction of β cells. This has been shown to work in rodent animals and include for instance the expression of: IL-2 (Goudy et al., 2011), IL-10 (Goudy et al., 2001), or IL-4 in combination with glucagon-like peptide-1 or hepatocyte growth factor/NK1 (Gaddy et al., 2012). However, the translation of the animal experiments to human trials is constrained by the lack of markers with acceptable positive and negative predictive values to unequivocally identify and select appropriate patients for preventive therapy who are at risk (van Belle et al., 2011).

3.2.4.2 *Gene therapy to induce β-cell neogenesis and regeneration*

Restoration of β-cell mass could result from two distinct strategies, by targeting therapeutic genes to existing β-cell to induce its regeneration, or targeting exocrine pancreas or extra-pancreatic tissues to generate new β-cells by reprogramming. The successful of these strategies requires halting the autoimmunity against β-cells, if not, the new β-cells will also be destroyed.

The endocrine pancreas may be efficiently transduced using adenovirus or AAV vectors to transfer key genes that stimulates replication of the remaining β-cells or induces β-

cell neogenesis from precursor cells (Ayuso et al., 2006) (Jimenez et al., 2011). Examples of this include gene transfer of insulin growth factor I (George et al., 2002), hepatocyte growth factor (Barshes et al., 2005; Fiaschi-Taesch et al., 2008; Gaddy et al., 2012; Jimenez et al., 2011) and glucagon-like peptid-1 (Gaddy et al., 2012).

Another approach of β cell neogenesis is to generate neo-β cells in the liver by the expression of key transcription factors involved in the differentiation of pancreatic cells. Key genes that have been transferred include the Pancreatic duodenal homeobox-1 (Pdx-1), NeuroD, Neurogenin-3 (Ngn3), Betacellulin (Btc) and MafA (Ferber et al., 2000; Kojima et al., 2003; Sapir et al., 2005; Zalzman et al., 2005; Zalzman et al., 2003; Kaneto et al., 2005; Yechoor et al., 2009a; Yechoor et al., 2009b).

3.2.4.3. *Genetic manipulation of extra-pancreatic tissue to improve glucose homeostasis*

There are two main strategies for controling glucose homostasis by engineering extrapancreatic tissues, one involving insulin production and secretion, and the other modifying glucose uptake.

3.2.4.3.1. *Extrapancreatic insulin production*

The expression of insulin by extrapancreatic cells holds the advantage that these cells may not be a target of the immune system (Lipes et al., 1996). Early studies showed an improvement of hyperglycemia by implantating fibroblasts transfected with proinsulin in diabetic mice (Selden et al., 1987). Nevertheless, there are some limitations, because only β-cells have the required proteases necessary for proinsulin processing into mature insulin. To solve this, the proinsulin gene was modified to be able to be processed by other proteases, like for instance the furin endoprotease. Furin endoprotease recognizes a four

amino acid motif, which can be introduced in the union site between C-peptide and chains A and B of the proinsulin by genetic manipulation (Groskreutz et al., 1994; Simonson et al., 1996).

Extrapancreatic tissues like skeletal muscle or the liver are the main tissues that have been proposed as a source for a basal insulin production (Muzzin et al., 1997; Abai et al., 1999; Mas et al., 2006; Dong et al., 2002; Falqui et al., 1999; Gros et al., 1999; Martinenghi et al., 2002; Riu et al., 2002).

Skeletal muscle is a good candidate for a genetic manipulation aimed at correcting metabolic diseases because it constitutes a large, accessible and vascularised tissue in which insulin receptors are present in high quantity and that is the major site of insulin-responsive glucose disposal (Lamothe et al., 1998; Mingozzi et al., 2011; Rao et al., 1996; White et al., 1994). In diabetic rodents insulin gene transfer to the skeletal muscle has been able to control fasting hyperglycemia (Croze et al., 2003; Oh et al., 2006; Shaw et al., 2002; Yin et al., 2001). Moreover, in our laboratory, transgenic mice expressing the human insulin gene under the control of the promoter of the myosin light chain (MLC) were generated. When diabetes was induced with streptozotocin (STZ), transgenic mice showed fasting normoglycemia and normoinsulinemia and in fed conditions reduced hyperglycemia and increased insulinemia. Injection of low doses of soluble insulin restored normoglycemia in fed STZ-induced diabetic transgenic mice, while STZ-induced diabetic controls remained highly hyperglycaemic. These results indicated that skeletal muscle may be a key target tissue for insulin production and suggested that muscle cells secreting basal levels of insulin, in conjunction with insulin therapy, may permit tight regulation of glycemia (Riu et al., 2002).

3.2.4.3.2. *Glucose uptake increase by extrapancreatic cells*

Another stategy of gene therapy for type 1 diabetes centres on the genetic manipulation of extrapancreatic cells to increase glucose uptake.

One of these strategies is increasing the glucose phosphorylation. Glucokinase (Gck), also called hexokinase IV, is a liver enzyme that facilitates phosphorylation of glucose to glucose-6-phosphate. Gck expression is found in the liver, pancreas, in the neuroendocrine cells of the gut and in the hypothalamus of humans and most other vertebrates (Matschinsky, 1996; Matschinsky et al., 2006). In each of these organs it plays an important role in the regulation of carbohydrate metabolism by acting as a glucose sensor and triggering shifts in metabolism or cell function in response to rising or falling levels of glucose. In skeletal muscle the rapid conversion of glucose to glucose-6-phosphate is catalyzed by HKI and II (Iynedjian, 1993; Printz et al., 1993c). HKI is widely and constitutively expressed, whereas HKII is the muscle and fat specific, insulin-stimulated isoform. Skeletal muscle GLUT4 translocation to the plasma mebrane and HKII mRNA levels and enzymatic activity are decreased when circulating insulin is low or in insulin resistance state (Printz et al., 1993a; Postic et al., 1994; Vestergaard et al., 1995). In contrast to HKII and all the other hexokinases, Gck has two important kinetic properties: (1) it has a high *Km* (lower affinity) for glucose (5-8mM), so Gck changes conformation and is increasingly more active in parallel with rising glucose concentrations in the physiologically important range of 4-10 mmol/l (72-180 mg/dl) (Bell et al., 1996; Matschinsky, 1996); and (2) its activity is not inhibited by its product, glucose-6-phosphate at physiological concentrations (Bell et al., 1996; Iynedjian, 1993; Printz et al., 1993c). These characteristics confer on Gck a higher glucose phosphorylation capacity at high glucose concentrations and the ability to control the rate of glucose uptake in relation to circulating concentrations (Matschinsky, 1996; Matschinsky et al., 2006; Printz et al., 1993c). During diabetes, Gck gene expression and enzyme activity

are low, and thus the liver is unable to metabolize blood glucose (Iynedjian, 1993; Printz et al., 1993c).

It has been shown that in transgenic mice overexpressing HKII or HKII plus GLUT1 in the skeletal muscle, no net effect on glucose tolerance or insulin action was detected (Chang et al., 1996; Hansen et al., 2000). Moreover, it has been described that overexpression of GLUT4 in skeletal muscle of STZ-induced diabetic transgenic mice led to a mild decrease in hyperglycemia, with no further improvement in a transgenic mice overexpressing GLUT4 plus HKII, probably because of HKII inhibition by glucose 6-phosphate (Lombardi et al., 1997).

In our laboratory transgenic mice expressing Gck under the control of the liver specific PEPCK promoter were generated to study whether the return of the expression of Gck in the liver of diabetic mice might prevent metabolic alterations. In contrast to STZ-induced diabetic control mice, diabetic STZ-induced transgenic mice showed strong reduction of hyperglycemia and a normalization of circulating ketone bodies, triglycerides, and free fatty acids (Ferre et al., 1996). Another group showed that adenoviral hepatic overexpression of Gck combined with subcutaneous insulin injections in STZ-induced diabetic rats provided better glycemic control than treatment with insulin alone (Morral et al., 2002). However long-term Gck overexpression in the liver of transgenic mice led to hyperglycemia, progressive glucose intolerance, increased intrahepatic trygliceride content, hyperinsulinemia, hepatic steatosis and increased triglyceride levels. Thus, liver Gck overexpression may have deleterious effects that depend on the level and the duration of the increase in Gck activity (Ferre et al., 2003; Morral et al., 2002). Skeletal muscle was proposed as an alternative tissue for Gck expression. For this reason, in our laboratory, transgenic mice expressing Gck in the skeletal muscle were generated. Healthy mice showed a decrease in fed and fasted glycemia, body weight maintenance, lower insulinemia and higher blood glucose disposal after an intraperitoneal glucose tolerance test. When

transgenic mice were made diabetic, they showed a partial hyperglycemia reduction and body weight maintenance (Otaegui et al., 2000). However, the lack of insulin in STZ-induced diabetic transgenic mice resulted in a failure to increase GLUT4 levels regarding to STZ-induced diabetic control mice (Burcelin et al., 1993; Napoli et al., 1995) and in insulin-dependent glucose transport (Kahn, 1992). However, Gck expression in STZ-induced diabetic transgenic mice led to higher muscle glucose 6-phosphate content and to increased muscle glucose disposal probably due to an increased insulin-independent glucose transport, which explains the partial improvement in hyperglycemia (Otaegui et al., 2000). Moreover, transgenic mice had a faster and stronger hypoglycemic response to insulin compared to STZ-induced diabetic control mice, indicating that transgenic mice were more sensitive to the hormone treatment (Otaegui et al., 2000). In another approach from our laboratory, differentiated myoblast cells expressing Gck showed a glucose-dependent increase in glucose uptake and utilization *in vitro*. When myoblasts were transplanted into muscles of STZ-induced diabetic mice, transplanted recipients showed a reduction in the hyperglycemia compared to recipients transplanted with wild type cells (Otaegui et al., 2002).

3.2.4.3.3. *Skeletal muscle glucose uptake increase combined with constitutive basal insulin production.*

In our laboratory double transgenic mice that specifically express Ins and Gck in skeletal muscle were generated. Double-transgenic mice prevented diabetic hyperglycemia development both in fed and fasted conditions and of the metabolic alterations when transgenic mice where made diabetic with STZ. This result demonstrated a synergic action between the constitutively basal insulin produced and the increased glucose uptake and phosphorylation by Gck. Furthermore, STZ-induced diabetic transgenic did not show hypoglycemia after an overnight fast. Also, compared with diabetic controls, which presented low levels of circulating insulin, transgenic mice showed increased insulinemia, that together with decreased glycemia led to an increase in Gck and a decrease in PEPCK gene

expression in the liver of transgenic mice which may have increased glucose uptake and decreased glucose production by the liver, thus contributing to the maintenance of normoglycemia. Furthermore fed STZ-induced diabetic transgenic mice showed restored glycogen levels similar to those of healthy controls (Mas et al., 2006).

These findings suggested that Ins and Gck might be expressed in skeletal muscle, using AAV1 vectors as a new gene therapy approach for T1D. To this end, AAV1-Ins and AAV1-Gck vectors were intramuscularly injected to *tibialis cranealis*, *gastrocnemius*, and *quadriceps* muscles of both hindlimbs to diabetic mice. Both genes were expressed under the control of CMV promoter. Treated mice showed a restoration and manteinance of normoglycemia in fed and fasting conditions for more than 4 months after treatment. In contrast, fed diabetic mice treated with AAV1-Ins alone, normalized fasted hyperglycemia but not fed hyperglycemia. Furthermore, Ins and Gck-treated diabetic mice showed normalization of metabolic parameters, glucose tolerance and food and fluid intake. Therefore, the joint action of basal insulin production and Gck activity generated a “glucose sensor” in the skeletal muscle that allowed a tight regulation of glycemia in diabetic mice (Mas et al., 2006).

The next step towards a clinical development of this therapy is to evaluate the feasibility of the approach in a large animal model of diabetes.

4. THE DOG AS A LARGE ANIMAL MODEL OF DIABETES

Historically, the dog has had an important role in diabetes research, because diabetes was first produced experimentally in a dog and that a role of the pancreas in the disease was thereby recognized (Engerman et al., 1982). The beneficial effects of insulin-containing extracts were also first demonstrated in a dog (Banting et al., 1922). More recently, diabetic dogs have been used to validate various insulin delivery systems, to test several insulin preparations (Goriya et al., 1978) and to apply pancreatic implant testing models (Sullivan et al., 1991). Furthermore, diabetic dogs commonly develop secondary complications resulting from diabetes or its treatment that include: blindness and anterior uveitis resulting from cataract formation; diabetic retinopathy (retinal hemorrage, microaneurisms); chronic pancreatitis; recurring infections; hypoglycemia; and ketoacidosis (Table 3) (Ettinger et al., 2010). However, most of the chronic complications of human diabetes require years (more commonly decades) to develop and become clinical, so, they are uncommon in diabetic dogs (Ettinger et al., 2010).

Table 3. Complications of Diabetes Mellitus in Dogs.

Complications of Diabetes Mellitus in Dogs	
Common	Iatrogenic hypoglycemia
	Persistent polyuria, polydipsia, and weight loss
	Cataracts
	Anterior Uveitis
	Bacterial infections, especially in the urinary tract
	Pancreatitis
	Ketoacidosis
	Hepatic lipidosis
Uncommon	Peripheral neuropathy
	Glomerulonephropathy, glomerulosclerosis
	Retinopathy
	Exocrine pancreatic insufficiency
	Gastric paresis
	Diabetic diarrhea

Adapted from Ettinger et al., 2010.

However, the only currently available research models of diabetes in dogs are experimentally induced and not arising genetically. Indeed, large animl models of autoimmune or spontaneous diabetes do not exist for research purposes in any specie.

Experimentally induced diabetes in dogs can be produced using different methods that include:

- Pancreatectomy. This can be partial or total. Total pancreatectomy guarantees diabetes development, but involves removal of exocrine pancreas too, so supplementation of the diet with pancreatic enzymes after the operation is essential (Engerman et al., 1982)

- Chemical induction. This can be performed with alloxan, streptozotocin or combining both. Alloxan and streptozotocin are the most prominent diabetogenic chemicals in

diabetes research and both are cytotoxic glucose analogues. Although their cytotoxicity is achieved via different pathways, their mechanisms of β-cell selective action are identical, accumulating in pancreatic beta cells via the GLUT2 glucose transporter (Lenzen, 2008). In the majority of the studies, the technique employed to induce diabetes utilized both pancreatic β-cell cytotoxic agents (Anderson et al., 1993). When administered singly, the required diabetogenic doses of either alloxan or streptozotocin were commonly associated with severe adverse effects on other organ systems, specially on the liver and the kidney (Rerup, 1970). The combination of alloxan and streptozotocin was demonstrated to disminish secondary effects because lower doses of both drugs can be used (Issekutz et al., 1974; Anderson et al., 1993; Liu et al., 2000). A mixed method has also been developed and consists in a 90% pancreatectomy together with the administration of a very low dose (2 mg/kg) of STZ perfused into the pancreaticoduodenal artery (Morales et al., 2005)

- Other techniques including administration of certain hormones like growth hormone, progestagens or benzothiadiazine derivatives have also been reported (Engerman et al., 1982)

In companion diabetic dogs, the primary goal of insulin therapy is the elimination of owner-observed signs occurring secondary to hyperglycemia and glycosuria. Limiting blood glucose concentration fluctuations and maintaining near-normal glycemia will help minimize clinical signs and prevent the complications of poorly controlled diabetes (Nelson, 2010). Serum fructosamine concentrations are used in diabetic dogs as a marker of mean blood glucose concentration over the previous 3 weeks and to assist in the diagnosis and monitoring of diabetic patients (Davison et al., 2005; Jensen, 1992; Reusch et al., 1993; Thoresen et al., 1997; Marca et al., 2000; Nelson, 2010). As in humans, it must be balanced the benefit of tight glucose control obtainable with aggressive insulin therapy against the risk

of hypoglycemia. Many insulin preparations have been shown to be effective in the treatment of canine diabetes mellitus (Church, 1981; Horn et al., 2000; Monroe et al., 2005). However, nowadays NPH insulin and pork lente insulin, both intermediate-acting insulins, are the most commonly insulins used in dogs (Behrend, 2006; Mori et al., 2008; Nelson, 2010). Insulin therapy is usually begun with NPH or pork lente insulin with an initial dose of 0,25 UI/kg every 12 hours (Mori et al., 2008; Nelson, 2010). It has been described that once-daily insulin administration in dogs results in very high insulin doses and an increased risk of hypoglycemia (Hess et al., 2000). Moreover, most of the dogs achieve better control of the disease when treated with twice-daily insulin administration (Horn et al., 2000; Monroe et al., 2005). A dietary therapy to help to reduce hyperglycemia is also usually initiated concurrently (Nelson, 2010). Recently, insulin glargine has emerged as a new option to treat canine diabetes. Initial studies demonstrated a duration of effect in dogs twice as long as that of NPH insulin, with a duration of action of approximately 18 to 24 hours, and a pronounced peak of action between 6 and 10 hours (Mori et al., 2008). For this reason, it can be administered every 24 hours instead of every 12 hours. However, in the few studies in which safety and efficacy of insulin glargine therapy has been evaluated, a 12-hour protocol has been used with an initial dose ranging from 0.25 to 0.5 UI/kg (Fracassi et al., 2012; Sako et al., 2010). In the most recent study, insulin glargine resulted similarly effective, in terms of the resolution of clinical signs, compared to previous studies using other insulins (Fracassi et al., 2012; Lorenzen, 1992; Monroe et al., 2005).

Recently gene therapy approaches that have been have been successfully translated into humans (Maguire et al., 2009; Nathwani et al., 2011) were preceded by convincing studies of efficacy in large animal models (Acland et al., 2001; Nathwani et al., 2006). Therefore, before reaching the clinical step, the diabetic dog can be a good model to assay new gene therapy approaches for diabetes.

III. OBJECTIVES

Diabetes is associated with severe secondary complications, caused largely by poor glycemic control. Treatment with exogenous insulin fails to prevent these complications completely, leading to significant morbidity and mortality. Skeletal muscle is responsible for the disposal of most (70%) of the circulating glucose after a meal. We hypothesized that regulation of glycemia could be achieved by generating a glucose sensor in the skeletal muscle through co-expression of glucokinase and low levels of insulin. We previously demonstrated that it is possible to generate a “glucose sensor” in the skeletal muscle through the co-expression of insulin (Ins) and glucokinase (Gck), increasing glucose uptake and correcting hyperglycemia in diabetic mice.

The *overall aim* of this study was **to evaluate the feasibility of the Ins and Gck gene therapy treatment for diabetes in a large animal model of the disease.**

This general aim was subdivided into two *specific aims*:

1. **To examine in diabetic dogs the effect of the expression in skeletal muscle of either Ins or Gck alone in the control of hyperglycemia.**

2. **To assess in diabetic dogs wether the expression of Ins and Gck was able to achieve long-term control of hyperglycemia and prevent the development of secondary complications.**

IV. RESULTS

1. GLYCEMIC CONTROL IN DIABETIC DOGS WITH EXOGENOUS INSULIN

Companion diabetic dogs are conventionally treated with long or intermediate-acting insulin analogs once or twice per day to counteract hyperglycemia and glycosuria (Nelson, 2010). The objective of conventional treatment is to limit blood glucose concentration fluctuations and to maintain near-normal glycemia in order to minimize clinical signs and to prevent the complications of poorly controlled diabetes (Nelson, 2010). To study the degree of glycemic control achieved in diabetic dogs treated with exogenous insulin treatment, experimental diabetes was induced in 3 dogs (DogDb1-3) with a single injection of STZ (30 g/kg) and of alloxan (40 g/kg) as previously described (Anderson et al., 1993).

1.1. Follow-up of the fasting glycemia and body weight

DogDb1 and DogDb2 quickly developed fasting hyperglycemia concomitantly with a gradual weight loss. One month after diabetes induction, DogDb1 and DogDb2 were highly fasting hyperglycemic and lost around 15% of body weight (Figure 1A and 1B). At this moment exogenous insulin treatment with a long-acting insulin analog (insulin glargine) was initiated in both dogs to reduce hyperglycemia and halt further weight loss. DogDb3 also quickly developed fasting hyperglycemia, but differently to DogDb1 and DogDb2, DogDb3 experienced a very accelerated weight loss, around 25%, of the initial body weight by day 4 after diabetes induction (Figure 1C). At this moment the insulin treatment was also initiated.

Despite insulin treatment fasting normoglycemia was not achieved in any of the dogs (Figure 1A-C), with one of the animals, DogDb3, experiencing severe fasting hyperglycemia (Figure 1C). However, exogenous insulin therapy prevented further weight loss in all dogs, although any of them never recovered its initial body weight (Figure 1A-C).

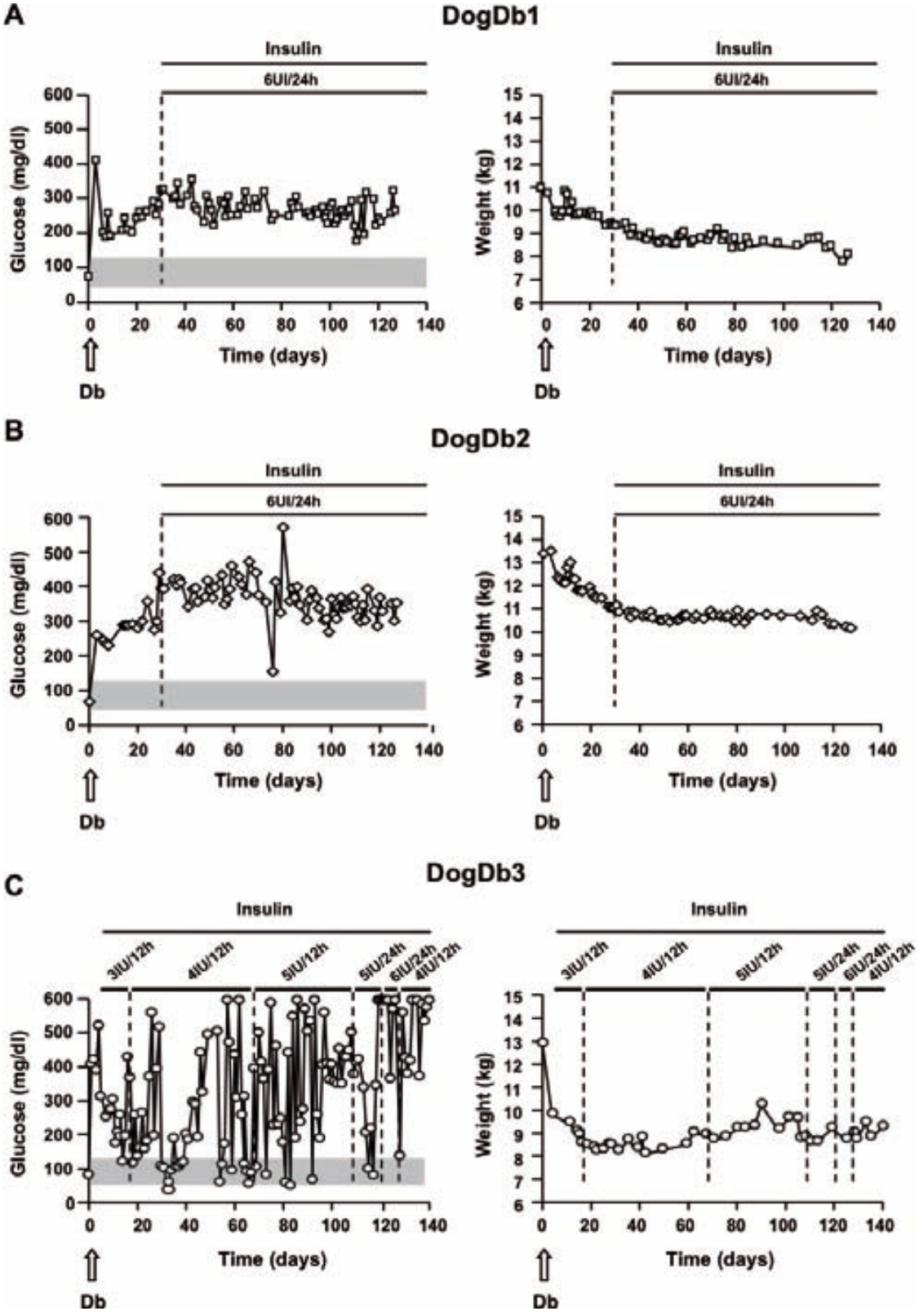

Figure 1. Glycemic control by exogenous insulin alone in control diabetic dogs. (A-C) Follow-up of fasting glycemia and body weight of diabetic control dogs (DogDb1-3) daily treated with exogenous insulin (dosage (IU) and timing (24h vs. 12h) is shown). Db, dog treatment with STZ and alloxan to induce diabetes. Grey bar indicates the fasting normoglycemia range in healthy dogs (Kaneko, 2008).

1.2. Serum fructosamine levels

Fructosamine is a compound that is formed when a carbonyl group of glucose reacts with an amino group of a protein, in a process called glycation. Affected proteins include albumin, the principal protein in the blood, other serum proteins, and hemoglobin, the major protein found inside red blood cells (RBCs). When glucose levels in the blood are elevated over a period of time, the glycation process is enhanced. These combined molecules persist for the life of the protein and provide a record of the average amount of glucose that has been present in the blood over that time period. Serum proteins have a lifespan of about 14 to 21 days, so glycated proteins, and the fructosamine determination, reflect the average glucose levels over a 2 to 3 week time period. Serum fructosamine concentration is not affected by acute increases in the blood glucose concentration, so it can be used to assist in the diagnosis and monitoring of diabetic patients (Jensen, 1992; Reusch et al., 1993; Thoresen et al., 1997; Marca et al., 2000; Nelson, 2010). Fructosamine is markedly elevated in diabetic dogs, serving as an indicator of poor glycemic control in veterinary medicine (Davison et al., 2005). In addition, normal fructosamine levels are a predictor that secondary complications will be delayed or not will be developed (Nelson, 2010).

Serum fructosamine was measured in DogDb1-3 regularly throughout the study. Despite exogenous insulin treatment, DogDb1-3 showed elevated fructosamine levels consistent with a poor glycemic control (>500 µmol/L) (Figure 2).

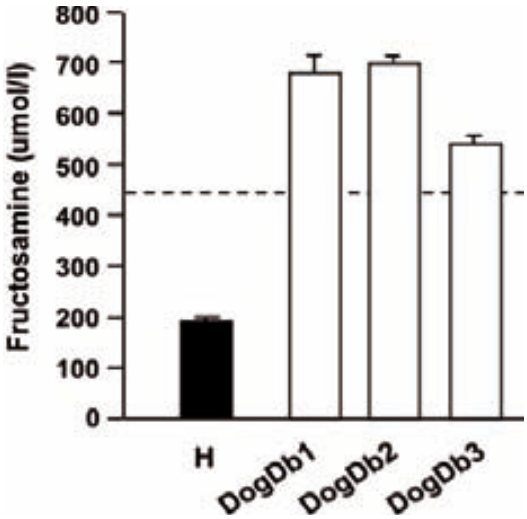

Figure 2. Serum fructosamine levels in control diabetic dogs. Fructosamine was determined in serum of 24-hour fasted dogs. 1C□□AP>OJN<HD □□PKK□MBHD□□□□□□HJGGAM□S>□GI OF>JI OMG?□B=□OND□?JBNDD?D<O?□ with dashed lines (Nelson, 2010). Fructosamine levels >500 µmol/L indicate poorly controlled diabetes (Davison et al., 2005; Nelson, 2010). Results are shown as 3 measurements/study period for DogDb1, 3 measurements/study period for DogDb2, 2 measurements/study period for DogDb3 and 35 measurements of healthy control samples (H).

1.3. Oral glucose tolerance tests

The oral glucose tolerance test (OGTT) measures the body's ability to metabolize glucose, or clear it out of the bloodstream and is one of the tests used to diagnose diabetes in human and veterinary medicine (American Diabetes Association, 2011; Kaneko, 2008). The dose used for glucose tolerance tests in adult humans is a solution that contains 75 g anhydrous glucose dissolved in water, and a dose of 1.75 g/kg adjusted for weight only in children younger than 16 years old (World Health Organization, 1999). Compared to healthy dogs that only showed a very slightly increase in blood glucose after the glucose load, DogDb1-3 showed a rapid rise in blood glucose remaining highly hyperglycemic throughout the duration of the test (Figure 3), with values over the limits of detection of the glucometer (600mg/dl) during all the test in DogDb3.

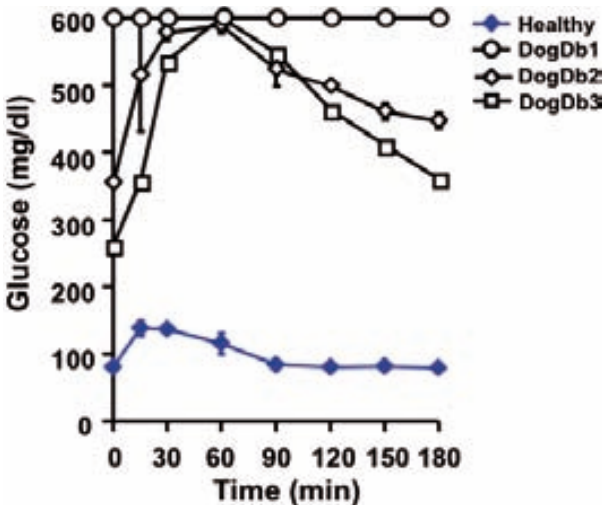

Figure 3. Oral glucose tolerance tests performed in control diabetic dogs. oGTT were performed at standard dose of glucose (1.75g/kg) in dogs before (Healthy) (mean±SEM, n=6) and after diabetes induction in DogDb1, DogDb2 and DogDb3 (mean±SEM, n=2).

Despite treatment with exogenous insulin, DogDb1-3 showed fasting hyperglycemia, they were unable to recover body weight and showed elevated serum fructosamine levels. This indicated that exogenous insulin treatment in diabetic dogs was unable to provide a good glycemic control.

2. GENETICALLY ENGINEERING OF THE SKELETAL MUSCLE IN DOGS

Skeletal muscle is a good candidate for a genetic manipulation aimed at correcting metabolic diseases because it constitutes a large, accessible and vascularised tissue in which insulin receptors are present in high quantity and is the major site of glucose disposal after a meal, taking up to 60-70% of circulating glucose in an insulin-dependent-manner, making it an important tissue in glucose homeostasis (DeFronzo et al., 1981; Lamothe et al., 1998; Mingozzi et al., 2011; Rao et al., 1996; White et al., 1994).

To achieve a widespread transgene expression in dog skeletal muscle, a preliminary study in a healthy dog was performed, in which an AAV1 that contained the green fluorescent protein (GFP) reporter gene under the control of the constitutive cytomegalovirus promoter was injected into the skeletal muscle of a healthy dog. A dose of $1x10^{12}$vg/kg of AAV1-GFP vector was injected to *gastrocnemius* and *tibialis cranealis* of the dog using a 5-point-needle to maximize the distribution of the vector (Figure 4A). Seven days after the vector injection, the dog was sacrificed and we observed a widespread expression of the GFP reporter gene in the area of the injected skeletal muscle (Figure 4B).

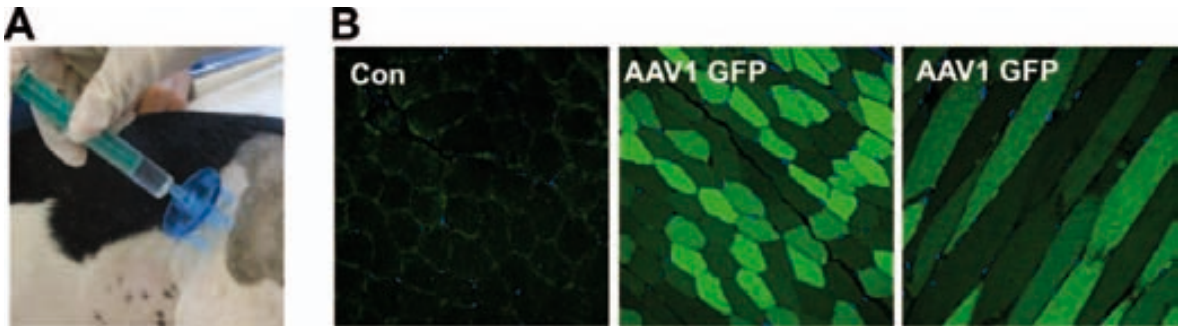

Figure 4. Efficient transduction of canine skeletal muscle with AAV1 vectors. (A) A 5-prong needle syringe was used as vector administration device. **(B)** Intramuscular delivery of the AAV1 vectors with this device results in efficient transduction of a large number of muscle fibers; representative images of GFP immunostaining are shown. Original magnification 200X.

3. GLYCEMIC CONTROL IN A DIABETIC DOG TREATED WITH AAV1-hIns

Previously in our laboratory, STZ-induced diabetic transgenic mice expressing the human insulin gene under the control of the promoter of the myosin light chain gene (MLC/Ins) showed fasting normoglycemia and normoinsulinemia and in fed conditions reduced hyperglycemia and increased insulinemia. These results indicated that skeletal muscle might be a key target tissue for basal insulin production to treat diabetes (Riu et al., 2002).

To study the degree of glycemic control achieved with the skeletal muscle expression of basal insulin levels in a diabetic dog, one dog was made diabetic and subsequently treated with AAV1-hIns (DogIns).

3.1. Follow-up of the fasting glycemia, body weight and insulinemia

After diabetes induction, DogIns developed fasting hyperglycemia (Figure 5A) and experienced a marked weight loss (around 20% from the weight prior to diabetes induction) (Figure 5B). Five days after diabetes induction DogIns was treated with $1x10^{12}$vg/kg of AAV1-hIns and fasting glycemia decreased to reach normoglycemia by around three months (Figure 5A). However, around 300 days after treatment, fasting glycemia values became slightly hyperglycemic and have since then showed periods of slight hyperglycemia combined with periods of normoglycemia (Figure 5A). Moreover, after treatment, DogIns recovered all the lost body weight after diabetes induction (Figure 5B) and has showed normoinsulinemia (Figure 5C) during all the length of the study (more than 4 years).

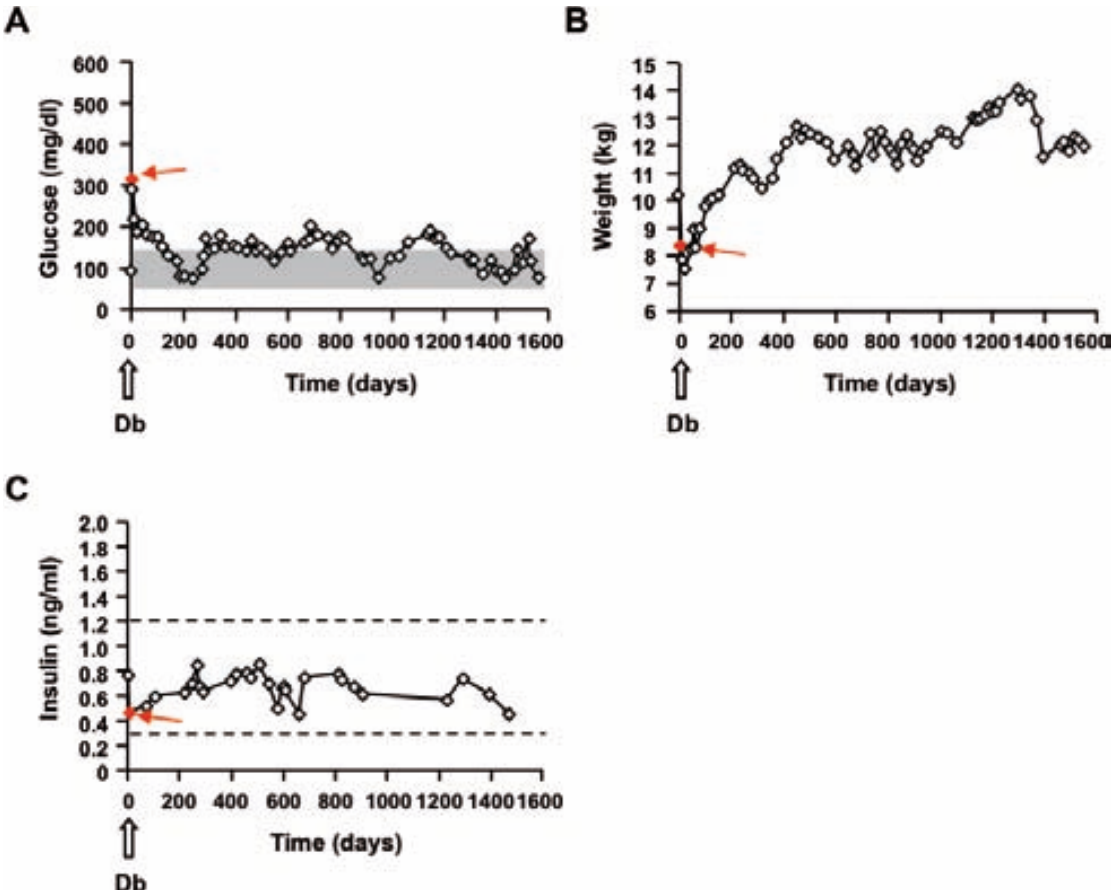

Figure 5. Glycemic control by AAV-hIns alone. (A,B) Diabetic dog treated with AAV1-hIns ($1x10^{12}$ vg/kg) (DogIns) showed partial normalization of fasting blood glucose levels **(A)**, and recovery of body weight loss (**B**). (**C**) Fasting insulinemia of DogIns. Dashed lines: average maximum and minimum fasting insulinemia values obtained by random measurements in 6 healthy dogs. Db, dog treatment with STZ+alloxan. Grey bar: fasting normoglycemia range in dogs (Kaneko, 2008).

3.2. Serum fructosamine levels

Fasting fructosamine was measured regularly throughout the study in DogIns. Conversely to DogDb1-3, AAV1-hIns treated dog always showed fructosamine levels within the range of those found in healthy dogs (Figure 6).

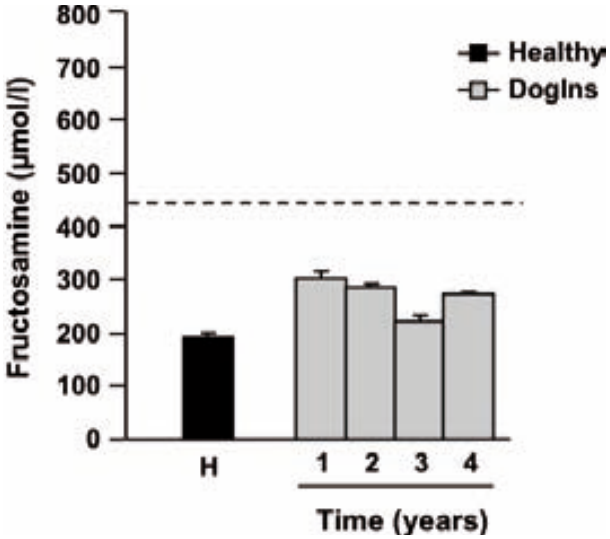

Figure 6. Serum fructosamine levels in dog treated with AAV1-hINS. Fructosamine was determined in serum of 24-hour fasted dog. 1C□AP>ON<HD□PKK□MGHD□□□□□HJGGAM□S>□GIOF>JIOMGG?□B=□ONID□?JBNID□ indicated with dashed lines (Nelson, 2010). Fructosamine levels >500 µmol/L indicate poorly controlled diabetes (Davison et al., 2005; Nelson, 2010). Results are shown as mean±SEM of >4 measurements/year for DogIns and 35 measurements of healthy control samples (H).

3.3. Oral glucose tolerance tests

DogIns were also subjected to OGTTs at a dose of 1.75 g/kg. Despite the presence of insulin, no major improvement was observed in the ability of DogIns to dispose of glucose (Figure 7).

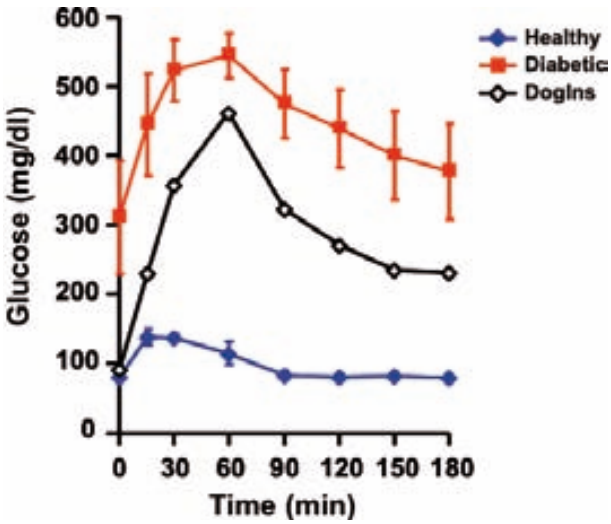

Figure 7. Oral glucose tolerance tests performed in dog treated with AAV1-hINS. OGTT were performed at standard dose of glucose (1.75g/kg) in DogIns before (Healthy) (mean±SEM, n=6), after diabetes induction (Diabetic) (mean±SEM, n=8) and after AAV1-hIns administration in DogIns (n=1).

Thus, skeletal muscle insulin expression was enough to achieve fasting normoglycemia, body weight recovery, establish insulin fasting levels in the range of a healthy dog and normalize serum fructosamine levels in a diabetic dog. However, DogIns showed a glucose disposal very similar to those showed by control diabetic dogs (DogDb1-3).

4. INS AND GCK GENE TRANSFER TO SKELETAL MUSCLE CORRECTS DIABETES IN DOGS

Previous results in our laboratory showed that diabetic mice treated with AAV1-hIns alone, normalized fasted but not fed hyperglycemia. However, Ins and Gck-treated diabetic mice showed a restoration and maintenance of normoglycemia both in fed and fasted conditions (Mas et al., 2006). Similarly, AAV1-hINS treatment of a diabetic dog restored normoglycemia in fast conditions but the ability of DogIns to dispose of glucose following OGTT was only modestly improved.

Then, we next examined the efficacy of genetic manipulation of skeletal muscle with both AAV1-Ins and AAV1-Gck (AAV1-Ins+Gck) to treat diabetic dogs in comparison with the exogenous insulin treatment and with AAV1-hIns administration alone.

4.1. Follow-up of the fasting glycemia, body weight and insulinemia

Five dogs were made diabetic and subsequently treated with AAV1-Ins+Gck. After diabetes induction Dog1 developed fasting hyperglycemia (>200 mg/dl) (Figure 8A) and experienced a marked weight loss (around 20% loss from the weight prior to diabetes induction) (Figure 8A). Furthermore, at this moment, no circulating insulin could be detected, taking into account that in diabetic dogs the insulin concentrations ranges from 0 to 0,2 ng/ml (Figure 8A) (Kaneko, 2008). One week after diabetes induction, Dog1 was treated with AAV1-hIns+rGck at a dose of $1x10^{12}$ vg/kg each vector. We previously demonstrated that this dose was able to normalize fasting and fed glycemia in mice (Mas et al., 2006). After treatment, this dog gradually returned to normoglycemia within 2 months (Figure 8A) and totally recovered the initial body weight (Figure 8A) in accordance with normalization of fasting insulin levels (Figure 8A). Since then, Dog1 has remained fasting normoglycemic with

a steady increase in body weight and healthy fasting insulinemia throughout the length of the study (more than 4 years).

At the same time a second dog (Dog2) was made diabetic and quickly developed fasting hyperglycemia (Figure 8B). Two months after diabetes induction, this dog had lost more than 30% of body weight (Figure 8B) concomitant with undetectable fasting insulin levels (Figure 8B). At this moment, Dog2 was treated with AAV1-hIns+rGck at a dose of $1x10^{12}$ vg/kg each vector. After treatment, fasting glycemia dropped sharply within 30 days (Figure 8B), coinciding with normalization of fasting insulin levels (Figure 8B) and a persistent weight gain (Figure 8B). Subsequent to this treatment, the dog has remained normoglycemic in fasting during the length of the study (more than 4 years).

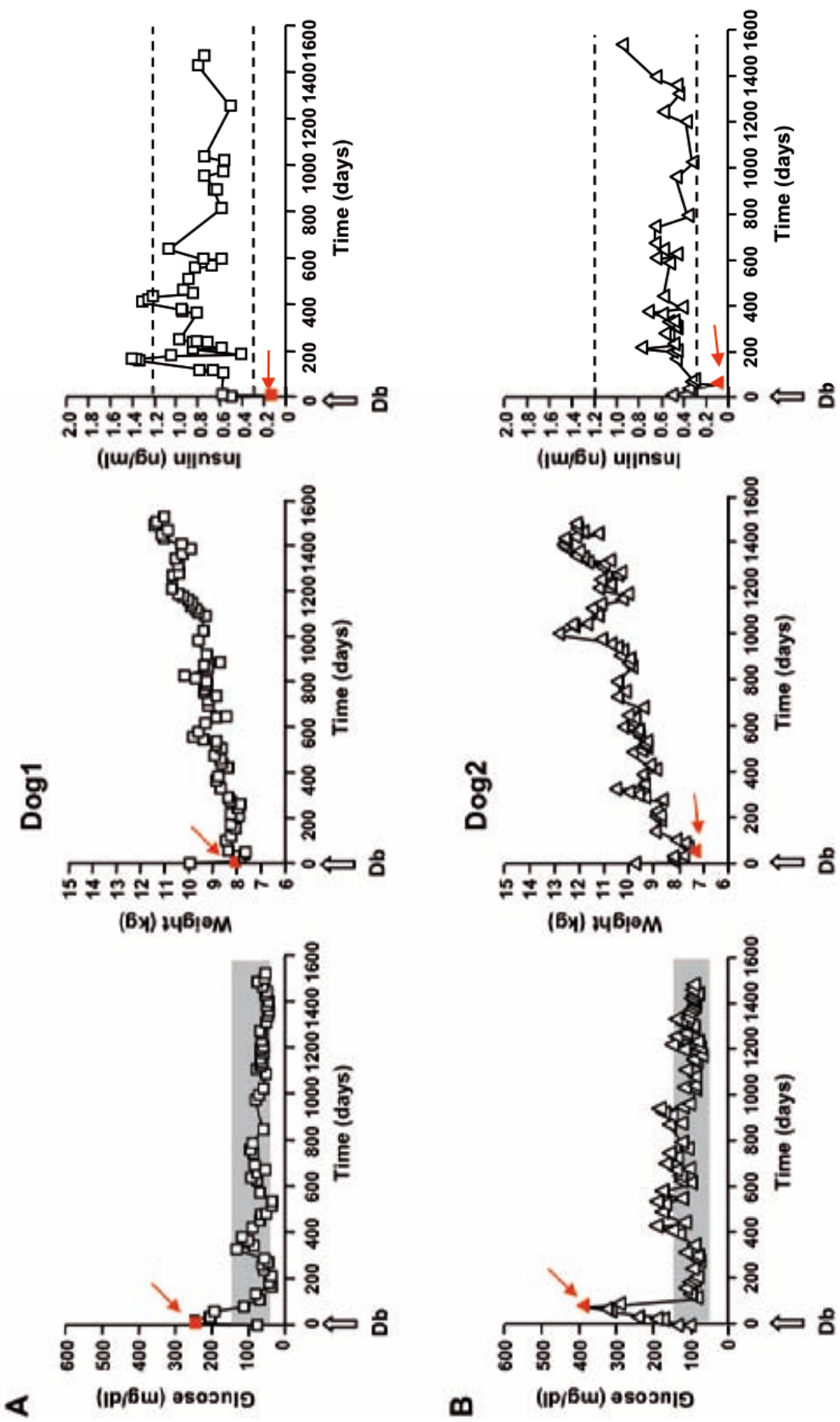

Figure 8. Treatment with AAV1-hIns and AAV1-rGck corrects diabetes in dogs. Follow up of glycemia, body weigth and insulinemia of two diabetic dogs, Dog1 **(A)** and Dog2 **(B)** treated with AAV1-hIns and AAV1-rGck vectors at $1x10^{12}$ vg/kg each. Dog1 and Dog2 had serum insulin levels that remained within the range of fasted healthy animals (dashed lines). Db, dog treatment with STZ+alloxan. Grey bars: fasting normoglycemia range in dogs (Kaneko, 2008).

To determine whether vector dose escalation was safe and could result in better glycemic control, two new diabetic dogs (Dog3 and Dog4) were treated with a higher dose ($2x10^{12}$ vg/kg) of AAV1-hIns+rGck. Dog3 was made diabetic and developed fasting hyperglycemia (Figure 9A), losing around 15% of body weight (Figure 9A) and showed a drop of fasting insulin levels (Figure 9A). Two weeks after diabetes induction, Dog3 was treated with AAV1-hIns+rGck at a dose of $2x10^{12}$ vg/kg for each vector. After treatment Dog3 returned to fasting normoglycemia in around three weeks (Figure 9A) and totally recovered the initial body weight (Figure 9A). Fasting normoglycemia with no episodes of hypoglycemia and stable body weight maintenance were observed during the entire length of the study (more than 2 years).

Despite efforts in adjusting the dose and timing of insulin treatment in DogDb3, this dog was very poorly controlled with exogenous insulin treatment (Figure 1C). For this reason, 5 months after diabetes induction, DogDb3 was treated with a dose of $2x10^{12}$ vg/kg of AAV1-hINS+rGck ,now renamed Dog4, resulting in normalization of fasting glycemia around one month after treatment and the cease of the need for exogenous insulin therapy (Figure 9B). In addition, after AAV administration, Dog4 experienced a maintenance in body weight (Figure 9B). Furthermore, before AAV treatment, no circulating insulin could be detected in Dog4 (Figure 9B). However, after treatment, a normalization of fasting insulin levels was observed (Figure 9B). Thus, in Dog4, in contrast when it was treated with exogenous insulin, AAV1-hINS+rGck treatment was able to normalize fasting glycemia.

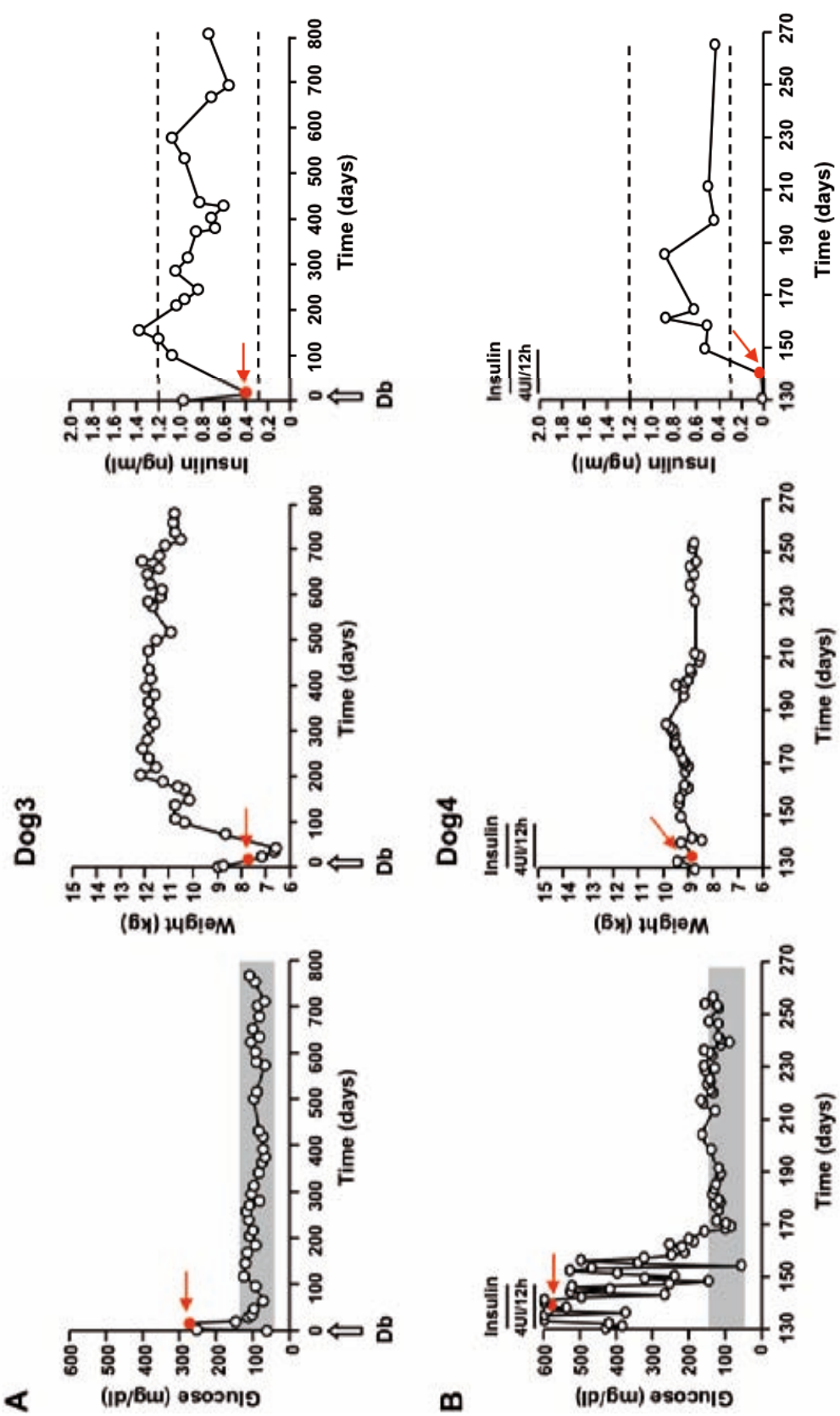

Figure 9. Treatment with AAV1-hIns and AAV1-rGck corrects diabetes in dogs. Follow up of glycemia, body weigth and insulinemia of two diabetic dogs, Dog3 **(A)** and Dog 4 **(B)** treated with AAV1-hIns and AAV1-rGck vectors at $2x10^{12}$ vg/kg each. Dog3 and Dog4 had serum insulin levels that remained within the range of fasted healthy animals (dashed lines). Db, dog treatment with STZ+alloxan. Grey bars: fasting normoglycemia range in dogs (Kaneko, 2008).

Codon-optimized versions of human transgenes were also tested at doses of $1x10^{12}$ vg/kg. To this end, a new dog (Dog5) was made diabetic and treated with AAV1-ohIns and AAV1-ohGck that led to rapid recovery of normoglycemia (Figure 10A), body weight (Figure 10B) and normoinsulinemia (Figure 10C) and, with no signs of fasting hypoglycemia detected during the five-month follow up.

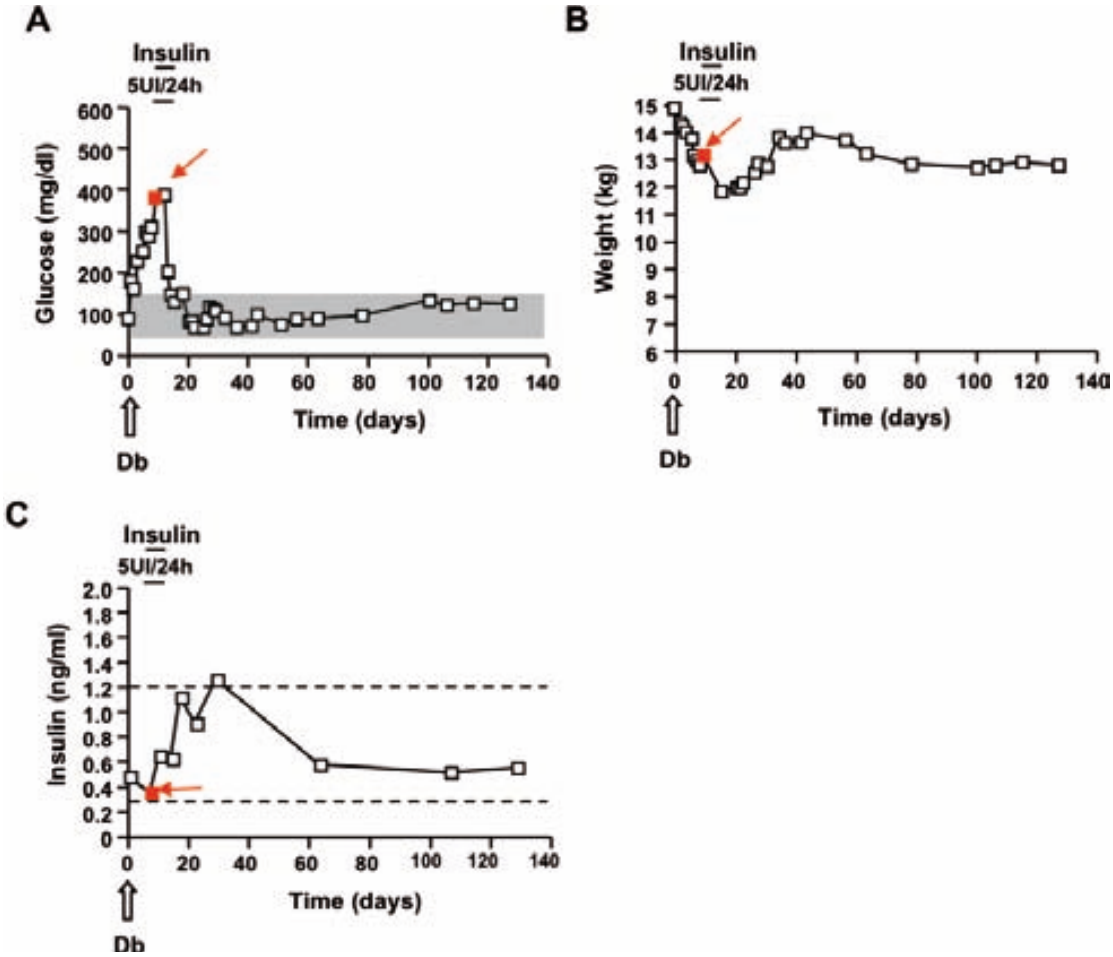

Figure 10. Treatment with AAV1-ohIns and AAV1-ohGck corrects diabetes in a diabetic dog. **(A,B)** Diabetic dog treated with AAV1-ohIns and AAV1-ohGck vectors at $1x10^{12}$ vg/kg each (Dog5) showed normalization of fasting blood glucose levels (**A**) and recovery of body weight loss (**B**). (**C**) Fasting insulinemia of Dog5. Dashed lines: average maximum and minimum fasting insulinemia values obtained by random measurements in 6 healthy dogs. Db, dog treatment with STZ+alloxan. Grey bar: fasting normoglycemia range in dogs (Kaneko, 2008).

4.2. Serum fructosamine levels

Fasting fructosamine was measured regularly throughout the study in AAV1-Ins+Gck treated dogs. Conversely to DogDb1-3 (Figure 2), AAV1-Ins+Gck treated dogs always showed fructosamine levels within the range of excellent diabetes control (Figure 11). DogDb3 showed elevated fructosamine levels when treated with exogenous insulin treatment (Figure 2). However, after treatment with AAV1-Ins and AAV1-Gck serum fructosamine levels in this dog (renamed Dog4) were normalized (Figure 11).

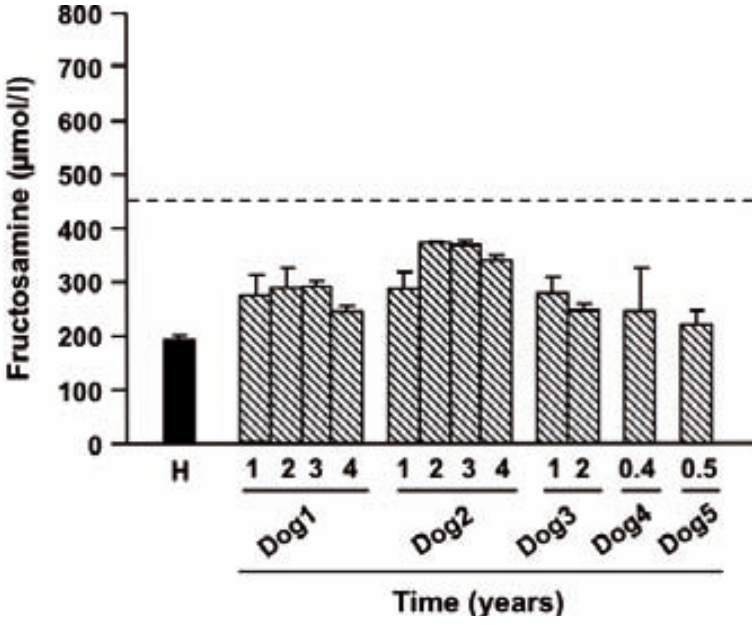

Figure 11. Serum fructosamine levels in dogs treated with AAV1-Ins+Gck Fructosamine was determined in serum of 24-hour fasted animals. 1C□□AP>JN<HD□□PKK□MBHD□□□□□□HJGGAM□S>□GI OF>JI OMG?□?B=□ONDD□ dogs is indicated with dashed lines (Nelson, 2010). Fructosamine levels >500 µmol/L indicate poorly controlled diabetes (Davison et al., 2005; Nelson, 2010). Results are shown as mean±SEM of >4 measurements/year or study period for Dogs-1-5 and 35 measurements of healthy control samples (H).

4.3. Oral glucose tolerance tests

When OGTT was performed at a dose of 1.75g/kg, treated dogs showed only a small rise in glycemia after the load, followed by return to normoglycemia within two hours, a profile considered non-diabetic by the American Diabetes Association (ADA) guidelines (2h plasma glucose <200mg/dl)(Figure 12A).

Furthermore, when performed at 3 g/kg, OGTT showed that combined Ins and Gck gene transfer in AAV1-Ins+Gck treated dogs, led to long-term and sustained improvement of glucose disposal, in particular, we documented a reduction of the peak of glycemia that usually occurred between 30 and 90 minutes after glucose administration as well as a 120 minutes glycemia below 200 mg/dl and a near-normalization in 180 minutes of the glucose value after oral glucose load compared to diabetic values (Figure 12B). These effects on glucose tolerance in both glucose doses were maintained even >4 years after AAV1-Ins+Gck treatment.

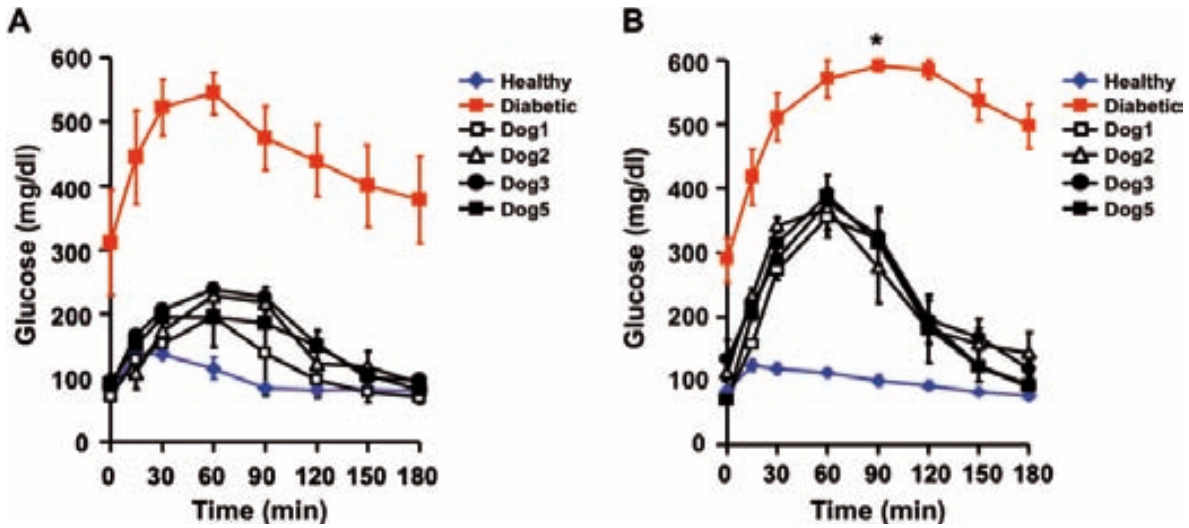

Figure 12. Oral glucose tolerance tests performed in dogs treated with AAV1-Ins+Gck. OGTT were performed in dogs before (Healthy) (mean±SEM, n=6) and after diabetes induction (Diabetic) (mean±SEM, n=8) and at several time points after AAV1-Ins+Gck administration in Dog1, Dog2, Dog3 and Dog5 (mean±SEM, n=2-3 OGTT performed every year or during the study period, in dogs with shorter follow-up)**. (A)** OGTT performed at a dose 1.75 g/kg. **(B)** OGTT performed at standard dose of glucose (3 g/kg).

4.4. Characterization of lipid profile

Poor metabolic control correlates with increased levels of total triglycerides in both diabetic humans and dogs. In contrast, in well-regulated diabetic patients, lipid levels are generally normal or even slightly decreased (Dullaart, 1995; Kaneko, 2008; Verges, 2009). Long-term follow up of serum triglycerides in AAV1-Ins and AAV1-Ins+Gck treated dogs depicted values in the normal range (figure 13A).

Free fatty acids released into circulation from the adipose tissue and its oxidation to ketone bodies in the liver is a consequence of inadequate insulin levels (Kitabchi et al., 2006; Miles et al., 1983). Ketone bodies are important clinically because of ketoacidosis that is often present when their plasma levels are high (Bruss, 2008). In AAV1-Ins and AAV1-Ins+Gck treated dogs free fatty acids (FFA) levels were similar to those of healthy animals (Figure 13B). Moreover ketone bodies were never detected in the urine of Ins and Gck treated dogs.

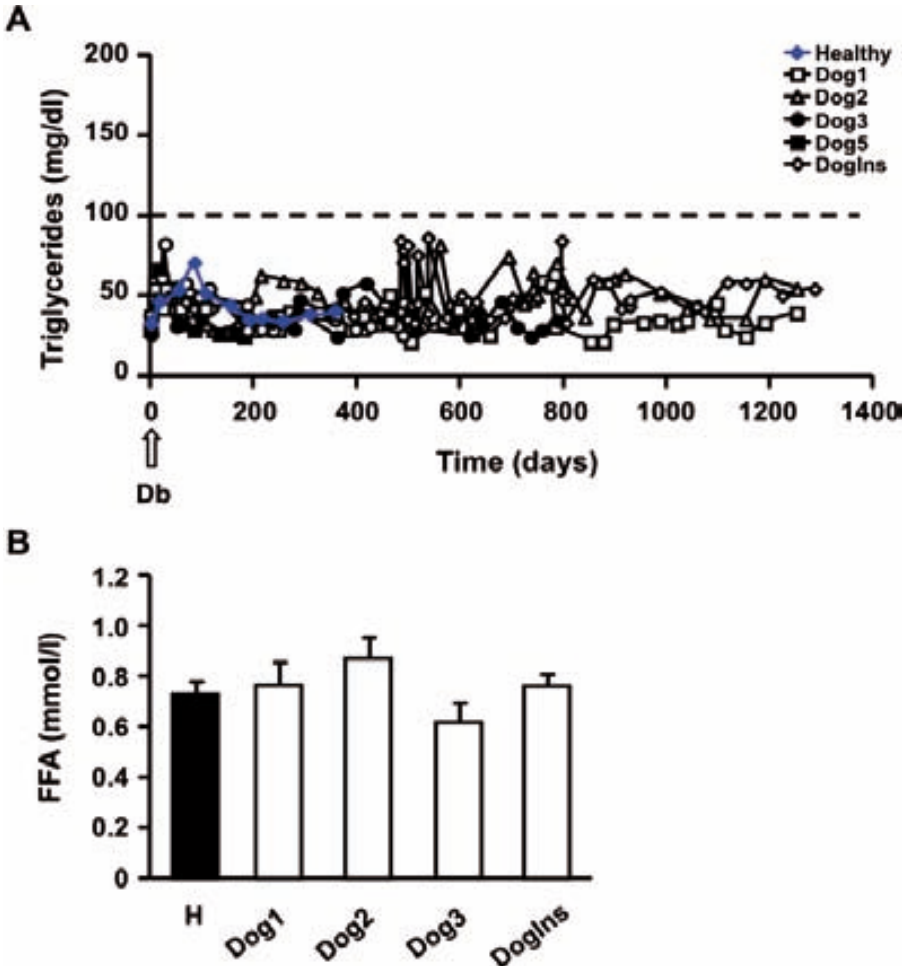

Figure 13. Serum triglycerides and Free Fatty Acid (FFA) are normal in AAV1-Ins and AAV1-Ins+Gck treated dogs. **(A)** Serum tryglicerides were determined in samples taken in 24-hour fasted dogs. Dashed line indicates upper limit for healthy dogs (Kaneko, 2008). **(B)** FFA levels were determined in samples taken in 24-hour fasted dogs. Results are shown as mean ± SEM (n=12-20 measurements per dog). H: healthy dog.

Thus, normal triglycerides, normal FFAs levels and the absence of ketones bodies in the urine suggested a good metabolic control in AAV1-Ins and AAV1-Ins+Gck treated dogs.

4.5. Skeletal muscle is the source of insulin

Long-term detection of insulin in the circulation was documented in all diabetic dogs after AAV treatment, at levels similar to those of endogenous insulin in fasting healthy animals.

To demonstrate that there was no significant contribution by the pancreas, a pancreas biopsy was taken from Dog1, Dog2 and DogIns nine months after AAV administration. In addition, necropsy samples were obtained from a healthy, a diabetic control dog and from Dog3 2.2 years after gene transfer. In all dogs, more than a 90% reduction in β-cell mass compared to a healthy dog was documented as measured by β-cell area/pancreas area (Figure 14A). Similarly, insulin and glucagon immunostaining of pancreatic sections of Dog3 demonstrated a nearly complete absence of β-cells (Figure 14B).

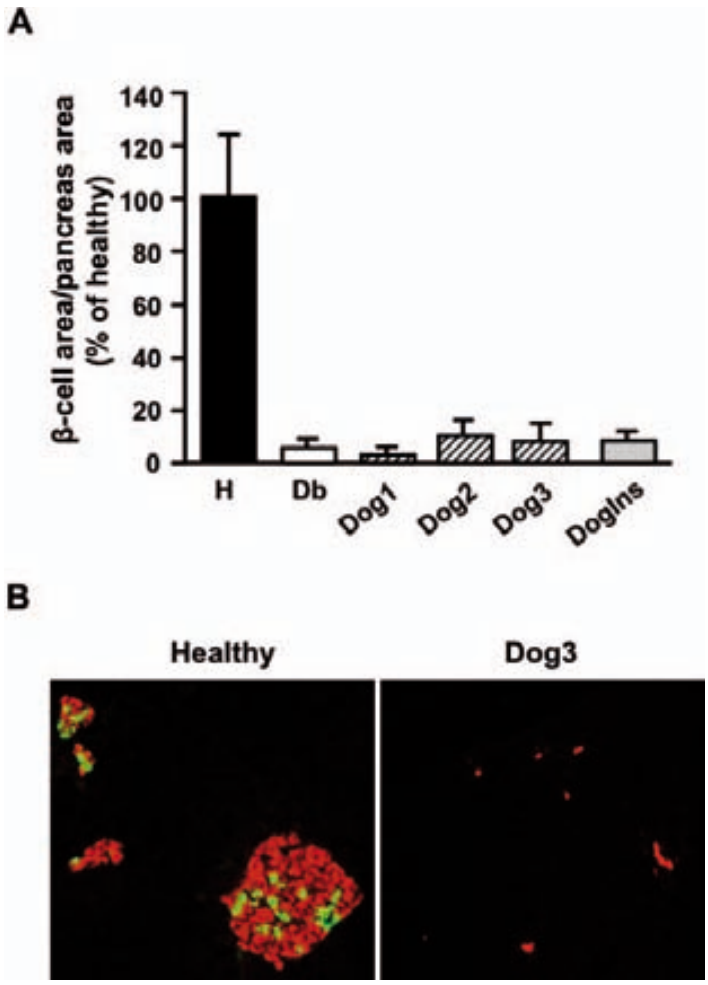

Figure 14. AAV treated dogs showed a near absence of β-cell mass. (A) Determination of the β-cell mass after diabetes induction. β-cell area was quantified in biopsy samples from Dog1, Dog2 and DogIns obtained 9 months after AAV administration. Necropsy samples from a healthy dog (H), a control diabetic dog (Db) and Dog3, sacrificed 2.2 years post-treatment were also analysed. **(B)** Representative images of pancreatic sections in Dog3 stained with insulin (red) and glucagon (green). Original magnification 200X.

To further confirm that the pancreas of AAV-treated dogs was unable to produce insulin after feeding, a time-course of insulin secretion after a meal was performed in Dog1, Dog2 and DogIns 4 years after treatment. In a time-course, insulin secretion test, only healthy dogs show the appearance of the first peak of insulin release that corresponds to pancreatic secretion of the insulin stored in secretory granules (Benthem et al., 2000). In contrast to healthy dogs, and in agreement with the lack of pancreatic insulin-producing cells, the first peak of insulin release after a meal was not observed in AAV-treated dogs (Figure 15). Instead, insulin levels maintained a steady peak despite the rise in glucose levels (data not shown), which is in agreement with stable, steady-state production and release of insulin from skeletal muscle.

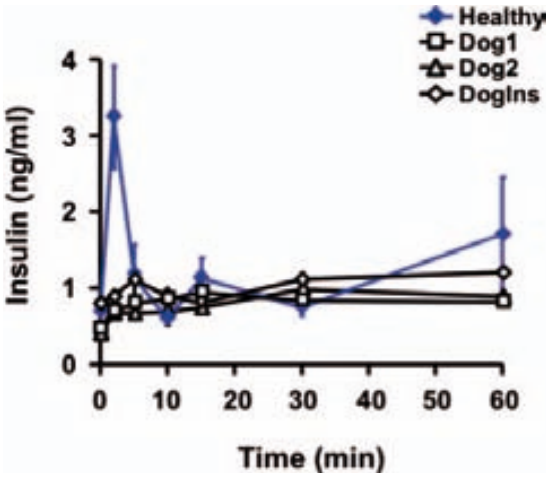

Figure 15. Time-course of insulin secretion after a meal revealed the absence of the first peak of insulin release. Tests were performed 4 years after treatment in Dog1, Dog2 and DogIns. Basal levels correspond to a 24-hour fasting period, after which animals were fed 30 g/kg of standard diet which was consumed by all dogs in less than 5 minutes, minimising variation from absorption. Insulin concentration was determined by RIA in serum samples taken from the cephalic vein at 2,5,10,15,30 and 60 minutes (n=2 healthy dogs and n=1 treated dogs).

To demonstrate that the skeletal muscle was the source of this circulating insulin, we studied if the skeletal muscle was expressing the hormone. Skeletal muscle biopsies where obtained from the treated quadriceps muscle of DogIns and Dog1, from a control non-injected healthy dog and necropsy samples where obtained of treated muscles from Dog3. Ins mRNA expression was detected in DogIns, Dog1 and Dog3. Gck mRNA expression was also determined and detected only in Dog1 and Dog3, as expected (Figure 16).

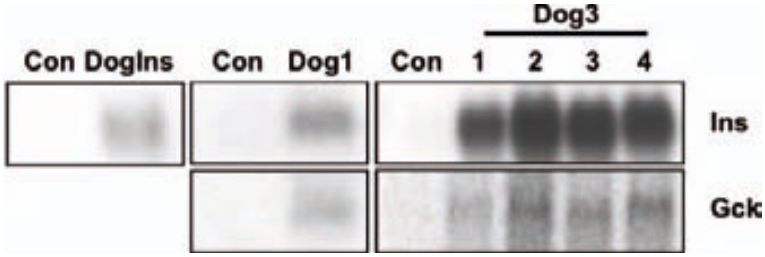

Figure 16. Transgene expression analysis. Northern blot of Ins and Gck mRNA in quadriceps biopsy specimens of DogIns, Dog1, and necropsy samples of Dog3, obtained 9 months and 2.2 years post treatment, respectively. Quadriceps biopsy of a healthy control dog was used as a control (Con).

C-peptide arises as a by-product of the enzymatic cleavage of proinsulin to insulin. In this process insulin and C-peptide are split from the prohormone and secreted into the portal circulation in equimolar concentrations (Horwitz et al., 1975). In the case of the proinsulin gene used in this study, it was modified to be able to be processed to mature insulin in the skeletal muscle by furin endoproteases. Furin endoproteases recognize a four aminoacids motif, which can be introduced in the union site between C-peptide and chains A and B of the proinsulin by genetic manipulation (Groskreutz et al., 1994; Simonson et al., 1996).

To determine if the insulin was correctly processed, we measured human C-peptide in all dogs before and after treatment (Figure 17). Human C-peptide was detected in all AAV1-Ins and AAV1-Ins+Gck treated dogs.

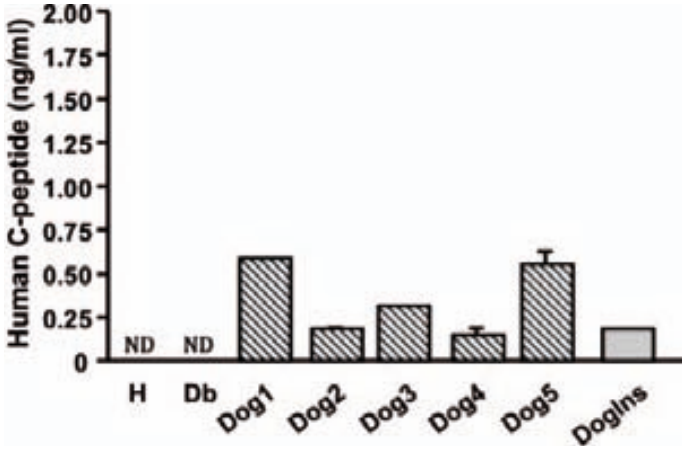

Figure 17. Human C-peptide was detected in all AAV-Ins and AAV1-Ins+Gck treated dogs. Human C-peptide was determined in 24-hour-fasting serum samples of Dog1, Dog2, Dog3, Dog4, Dog5 and DogIns regularly after treatment (n=23-56 measurements per dog). Values from healthy (H) and untreated control diabetic (Db) dog represent 20 and 24 samples respectively. Results are shown as mean ± SEM.

All these results together indicated that proinsulin was expressed, correctly processed to mature insulin and released into the circulation from skeletal muscle. Importantly, there was a negligible contribution from the pancreas to circulating insulin levels according to the absence of the first peak of insulin release after a meal and the more than 90% reduction of β-cell mass in treated dogs.

To analyze if the α-cell function was preserved after diabetes induction in AAV1-Ins and AAV1-Ins+Gck treated dogs, fasting glucagon serum was determined regularly. AAV1-Ins and AAV1-Ins+Gck treated dogs presented fasting glucagon levels similar to those of a healthy control dog, whereas in diabetic control dogs (Db) glucagon levels where higher (Figure 18).

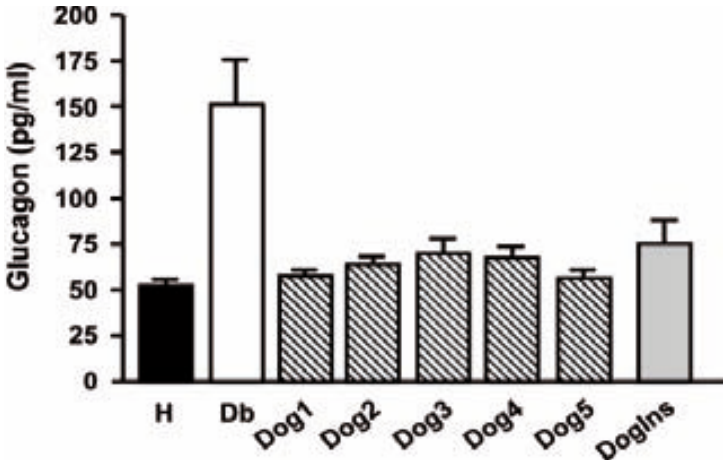

Figure 18. AAV1-Ins and AAV1-Ins+Gck treated dogs present normalized circulating glucagon levels. Glucagon was determined in 24-hour-fasting serum samples of Dog1, Dog2, Dog3, Dog4, Dog5 and DogIns regularly (n=5-13 measurements per dog). Values from healthy (H) and exogenous insulin treated control diabetic dogs (Db) samples represent 18 and 7 samples respectively. Results are shown as mean ± SEM.

4.6. Viral vector biodistribution studies

Dog3 was euthanized 2.2 years after gene transfer and vector genome biodistribution analysis was performed by qPCR assay in order to assess which organs had been transduced after the intramuscular administration of the AAV1 vectors. Consistent with previous findings for this serotype and route of administration (Leger et al., 2011; Toromanoff et al., 2008) most of the vector was found in skeletal muscle (Table 1). Some samples of the injected muscle revealed a high number of vector genomes whereas a few samples from injected muscles had low copy numbers, likely due to sampling of tissue more distant from sites of direct injection. Low vector gene copy numbers were detected in most peripheral tissues. Expression of insulin and glucokinase was undetectable in the liver by Northern and qPCR analysis (data not shown) despite the detection of vector genomes. This is likely due to silencing of the CMV promoter in liver (Al-Dosari et al., 2006).

Table 1. Biodistribution of AAV1 vector in Dog3 2.2 years after treatment.

Tissue	vg/diploid genome
Right Triceps brachii (untreated muscle)	0.21
Right Triceps brachii (untreated muscle)	0.11
Left Biceps femoris	0.05
Left Biceps femoris	0.03
Left Biceps femoris	5.68
Left Quadriceps femoris-Vastus lateralis	6.88
Left Semitendinosus	3.16
Left Tibialis anterior	1.57
Left Tibialis anterior	8.36
Left Tibialis anterior	15.14
Right Biceps femoris	0.18
Right Biceps femoris	0.02
Right Biceps femoris	0.75
Left Quadriceps femoris-Vastus lateralis	1.21
Right Semitendinosus	6.14
Right Extensor digitorum longus	1.35
Right Extensor digitorum longus	15.31
Right Extensor digitorum longus	2.38
Right Tibialis anterior	7.42
Kidney (cortex)	0.22
Kidney (medulla)	0.38
Lung (right middle lobe)	0.67
Lung (right caudal lobe)	0.61
Heart (left ventricle)	0.19
Heart (left atrium)	0.22
Gonads (testis)	0.04
Liver (left lateral lobe)	1.15
Liver (quadrate lobe)	1.12
Liver (right medial lobe)	0.40
Liver (right lateral lobe)	0.88
Liver (caudate process)	0.55
Liver (papillary process)	0.81
Pancreas (left lobe)	0.15
Pancreas (right lobe)	0.03
Spleen	0.57

Dog3, who received a vector dose of $2.0x10^{12}$ vg/kg of AAV1-hINS+rGck, was sacrified 2.2 years after administration and biodistribution determined by qPCR in tissue samples. Values represent individual samples.

4.7. Skeletal muscle morphology and glycogen content

To study the effects of long-term expression of Ins and Gck in the skeletal muscle, histological analysis and glycogen content determination of injected skeletal muscle were performed.

Normal muscle morphology without excess glycogen storage (glycogenosis) (Table 2 and Figure 19) was documented in the same biopsy samples used for muscle expression studies from DogIns obtained 9 months after treatment and in necropsy samples from Dog3 sacrificed more than two years after treatment. No signs of muscle pathology or inflammation were observed by hematoxylin and eosin (H&E) staining, or by laminin immunodetection. Periodic acid-Schiff (PAS)-staining showed normal glycogen storage.

Table 2. Skeletal muscle glycogen content determination after AAV1-Ins or AAV1-Ins+Gck gene transfer.

Dog	Glycogen (mg/g)
Con	3.31 ± 0.55
DogIns	0.910
Dog1	2.711
Dog3	2.76 ± 0.47

Skeletal muscle glycogen content was determined in necropsy samples from a healthy control dog (Con) and Dog3 (2.2 years post-treatment) and in muscle biopsies from DogIns and Dog1 obtained 9 months after AAV1 administration. Results are mean±SEM of 3 samples for healthy control dog and 4 samples obtained from different sites for Dog3. Results for DogIns and Dog1 correspond to a single biopsy sample.

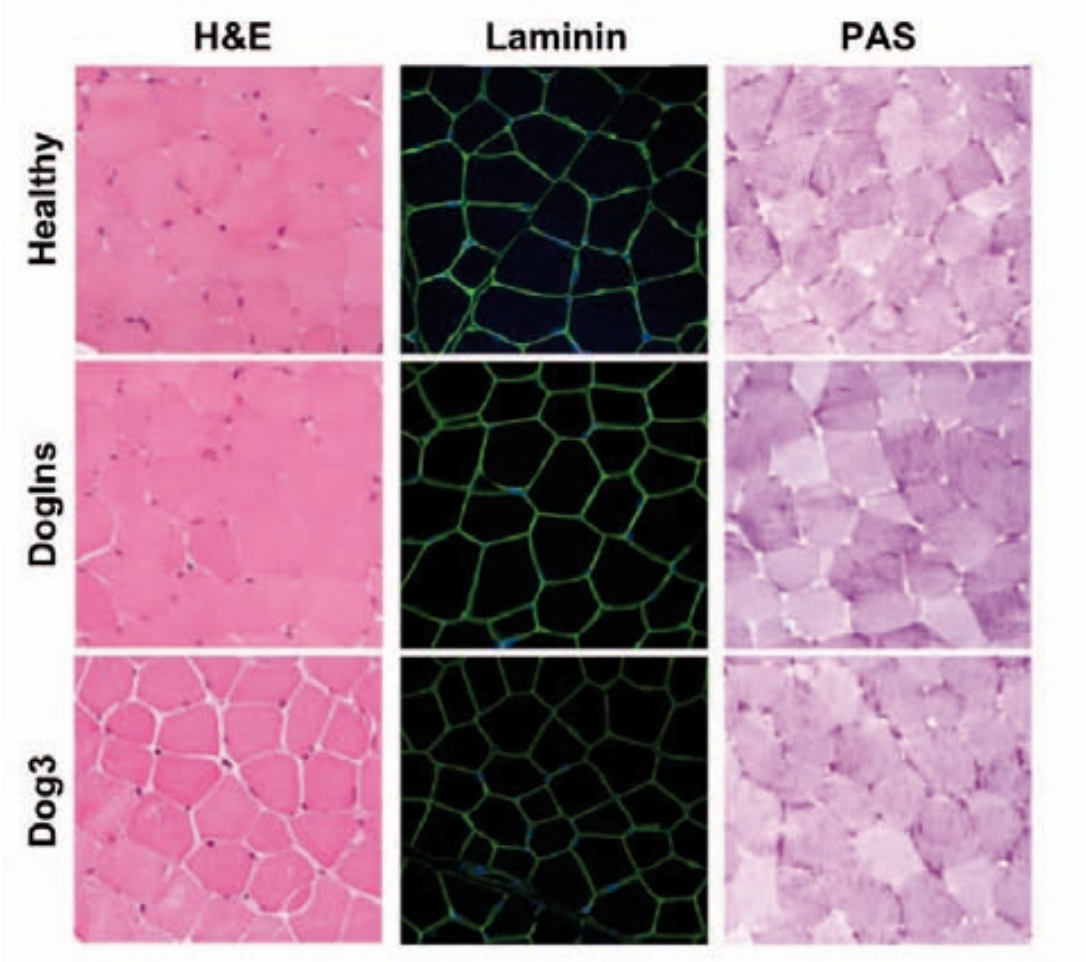

Figure 19. Morphologic analysis of muscle structure after AAV1-Ins or AAV1-hINS+Gck gene transfer. Morphologic analysis performed in skeletal muscle biopsies of DogIns obtained 9 months after AAV1 administration, in necropsy samples from Dog3 (2.2 years after treatment) and from necropsy samples of a healthy control dog. H&E staining and laminin immunodetection were performed to study muscle integrity. PAS staining was performed to evaluate glycogen content in the muscle samples. Original magnification 200X.

Thus, normal muscle morphology without abnormal glycogen accumulation was documented in the skeletal muscle of the AAV1-Ins and AAV1-Ins+Gck treated dogs, indicating that the long-term Ins and Gck expression from skeletal muscle did not induce pathological changes.

5. ROLE OF THE GCK TRANSGENE IN THE GLYCEMIC CONTROL

Previously in our laboratory, STZ-treated transgenic mice expressing Gck in the skeletal muscle showed a partial reduction of hyperglycemia and body weight maintenance (Otaegui et al., 2000). It is described that the lack of insulin in STZ-treated mice resulted in a failure to increase GLUT4 levels (Burcelin et al., 1993; Napoli et al., 1995) and in a failure in insulin-dependent glucose transport (Kahn, 1992). When transgenic mice were treated with insulin, they showed a faster and stronger hypoglycemic response to insulin compared to STZ-treated diabetic control mice, indicating that transgenic mice were more sensitive to the hormone treatment (Otaegui et al., 2000).

To study the effect on the control of diabetic hyperglycemia of skeletal muscle Gck expression alone in dogs, two diabetic dogs were treated with AAV1-ohGck (DogGck1 and DogGck 2)

5.1. Follow-up of the fasting glycemia, body weight, insulinemia and fructosamine levels

Two diabetic dogs were treated with AAV1-oGck vectors alone at $2x10^{12}$ vg/kg (DogGck1 and DogGck2). After vector delivery, both dogs remained hyperglycemic and required administration of exogenous insulin to reduce hyperglycemia and stabilize weight loss (Figure 20A and 20B), demonstrating that Gck expression alone is not sufficient to counteract hyperglycemia.

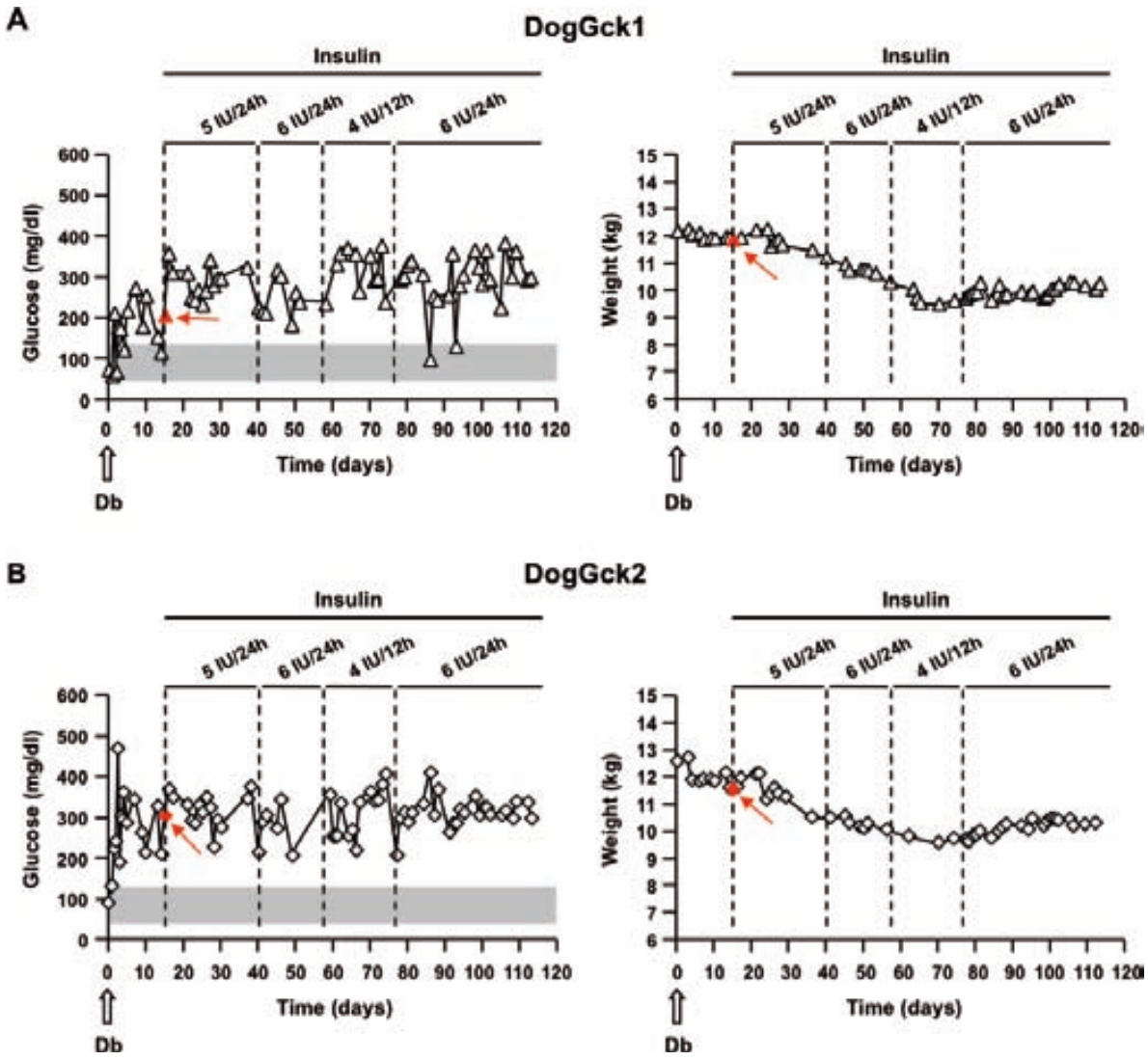

Figure 20. Treatment of DogGck1 and DogGck2 with AAV1-ohGck alone is not able to rescue diabetes (A,B) Follow up of glycemia and body weigth of DogGck1 **(A)** and DogGck2 **(B)** treated with AAV1-ohGck vectors at $2x10^{12}$ vg/kg each). Db, dog treatment with STZ+alloxan. Grey bars: fasting normoglycemia range in dogs (Kaneko, 2008).

Furthermore, fasting fructosamine levels were elevated in both, DogGck1 and DogGck2, compared with healthy values (Figure 21).

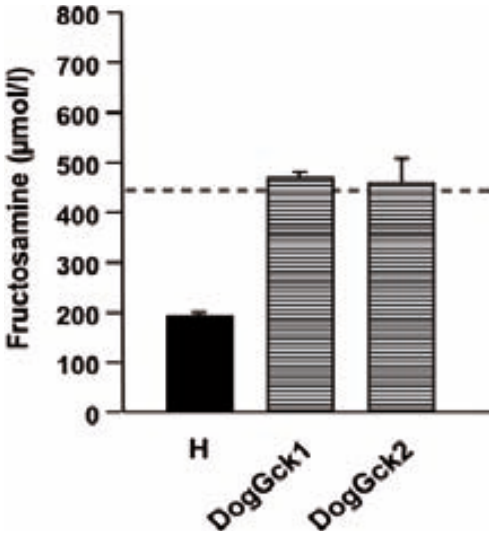

Figure 21. Serum fructosamine levels in dogs treated with AAV1-ohGck. Fructosamine was determined in serum of 24-hour fasted dogs. 1C□□AP>ON<HD□□PKK□MGHDO□□□□□□HJGGAVMS>□GI OF>JI OMG?□?B=□ONDO□ dogs is indicated with dashed lines (Nelson, 2010). Fructosamine levels >500 µmol/L indicate poorly controlled diabetes (Davison et al., 2005; Nelson, 2010). Results are shown as mean±SEM of 5 measurements/study period for Dog-Gck1 and Dog-Gck2 and 35 measurements of healthy control samples (H).

5.2. Oral glucose tolerance tests

Dogs expressing Gck alone showed impaired OGTT (1.75 g/kg), with glycemia curves similar to those of diabetic non-treated animals (Figure 22A). When OGTT was performed with simultaneous subcutaneous injection of insulin, both DogGck showed higher sensitivity to exogenous insulin than untreated control diabetic dogs, with glycemia dropping below 200 mg/dl in both animals (Figure 22B).

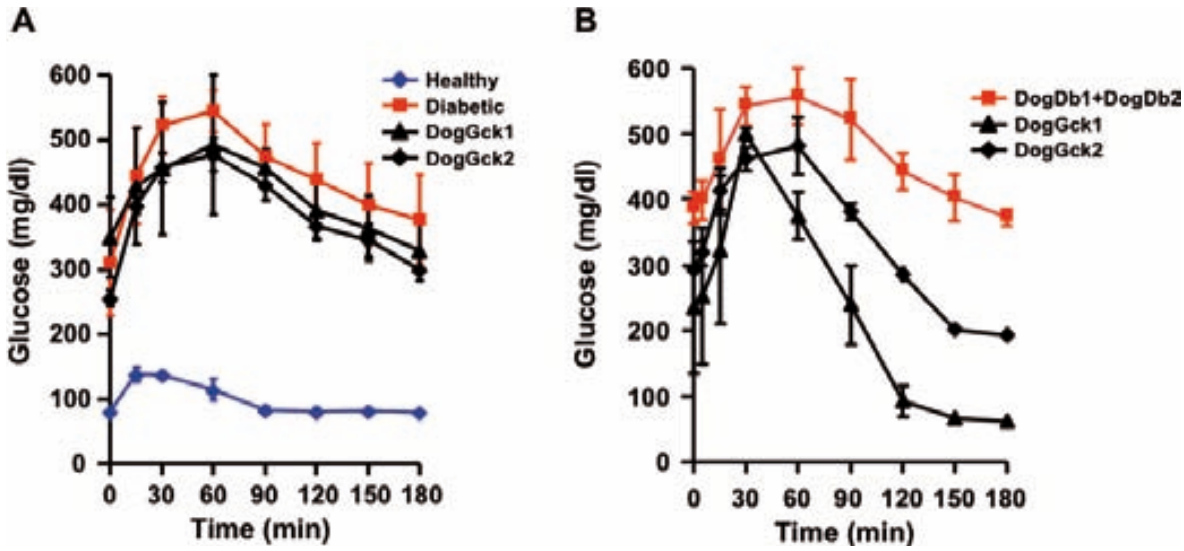

Figure 22. Oral glucose tolerance tests performed in dogs treated with AAV1-ohGck. (A) Oral glucose tolerance tests (OGTT) were performed at standard dose of glucose (1.75g/kg) in dogs before (Healthy) mean±SEM, n=6) and after diabetes induction (Diabetic) (mean±SEM, n=8) and at several time points after AAV1-ohGck administration in DogGck1 and DogGck2 (mean±SEM, n=3)**. (B)** AAV1-Gck treated dogs showed increased insulin sensitivity. DogGck1, DogGck2 and untreated control diabetic dogs (DogDb1 and DogDb2) were given a glucose load of 1.75g/kg together with a subcutaneous injection of insulin (6 IU of Lantus). Data are represented as mean ± SEM, n=2.

5.3. Expression of Gck in skeletal muscle of mice.

To examine whether an increase in Gck activity led to a higher insulin sensitivity in diabetic mice, diabetes was induced with STZ to 8 months-old C57Bl6 mice. Two weeks after diabetes induction, mice were highly hyperglycemic and, at this moment, $4X10^{12}$ vg/kg of AAV1-ohGck or AAV1-null were injected into the *quadriceps, gastrocnemius* and *tibialis* of both hindlimbs.

Despite the detection of Gck activity in AAV-ohGck treated mice (Figure 23A), no significant differences in fed glycemia between the group treated with AAV1-ohGck and the group treated with AAV1-null were observed (Figure 23B). To assess the insulin sensitivity of both groups, a dose of 0.75 UI/kg of a fast-insulin analog (Humulin regular) was injected into mice 3 months after AAV treatment and glycemia followed during 180 minutes. In contrast to injected diabetic null mice that remained highly hyperglycemic during all the experiment, Gck

expressing diabetic mice showed a marked reduction of glycemia (Figure 23C). Thus, Gck treated mice showed an increased hypoglycemic response to insulin demonstrating increased insulin sensitivity.

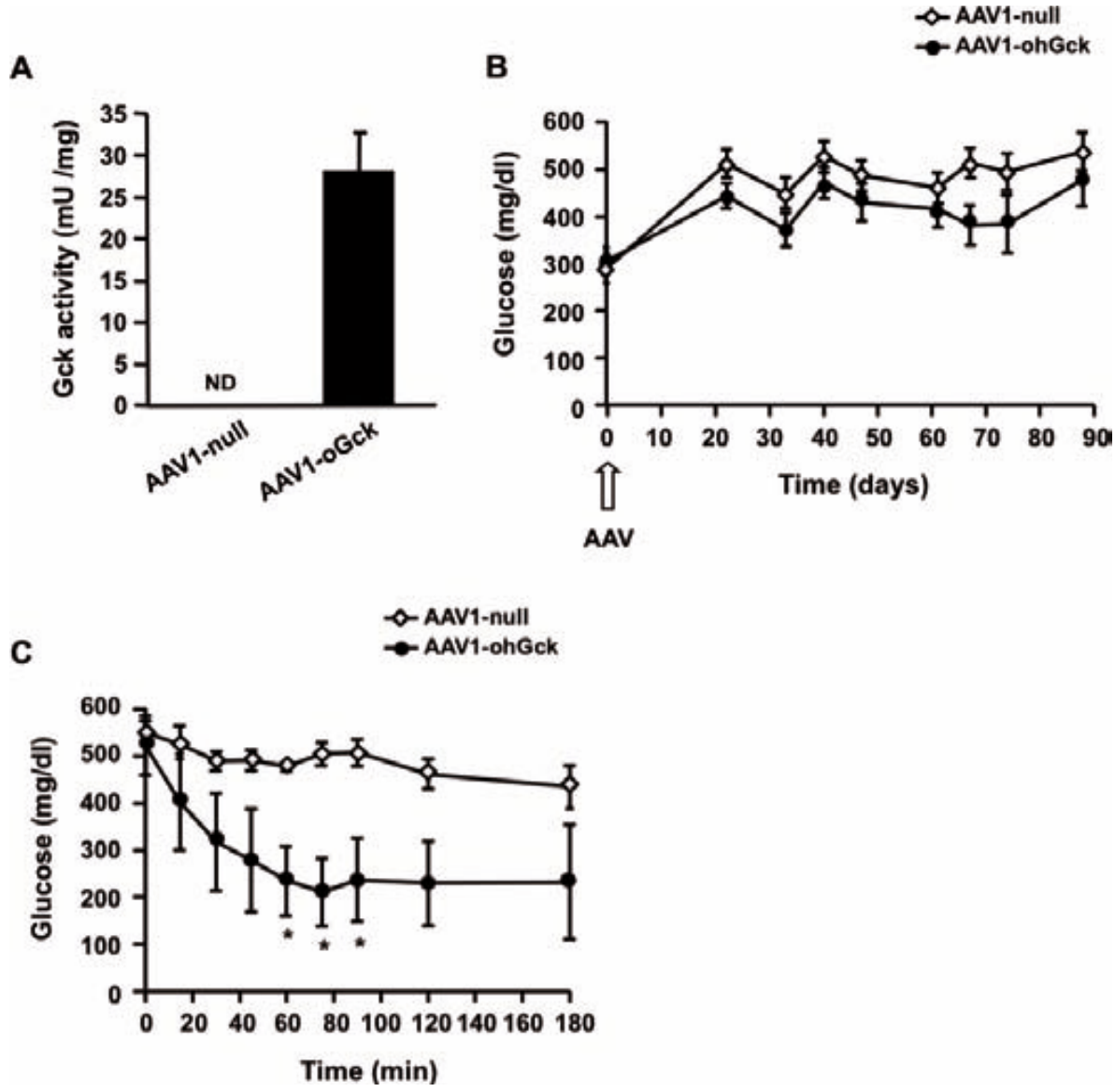

Figure 23. Expression of Gck in skeletal muscle increased insulin sensitivity. STZ-treated mice were intramuscularly administered with either AAV1-ohGck or AAV-null vectors ($4x10^{12}$ vg/kg). **(A)** Glucokinase activity was detected in the gastrocnemius of mice injected with AAV1-ohGck 3-months after vector administration. **(B)** Blood glucose profile of diabetic mice treated with AAV1-ohGck or AAV1-null vectors. Glycemia was determined in fed conditions in the absence of exogenous insulin treatment. **(C)** Insulin sensitivity was determined in diabetic mice 3-months after treatment with AAV1-ohGck or AAV1-null vectors. Fed mice were given an intraperitoneal injection of insulin (0.75IU/kg, Humulin regular, Eli Lilly) and glycemia was followed for 180 min. Results are expressed as the mean ± S.E.M. of n=12 mice per group. *p<0.05 vs AAV1-null.

6. EFFECTS OF EXERCISE IN TREATED DOGS

Human and dog diabetic subjects performing exercise are at risk of hypoglycemia or hyperglycemia, with an adjustment of insulin and carbohydrate intake necessary prior to exercise (De Feo et al., 2006; Nelson, 2010; Riddell et al., 2006). To assess how AAV-treated dogs performed during and after exercise, they were challenged with exercise under fasting conditions.

Dogs were 24-hour fasted and challenged by 37 minutes of progressive moderate exercise, consisting on running in a belt treadmill at increasing speed and slope. A drop in blood glucose was observed after exercise in DogIns, DogGck1 and DogGck2, because these animals had a baseline glucose level higher than all other Ins and Gck treated animals. Dog1-5 treated with AAV1-Ins+Gck showed good glycemic control under exercise, comparable to healthy dogs. Importantly, no dog developed hypoglycemia or hyperglycemia during or after exercise (Figure 24).

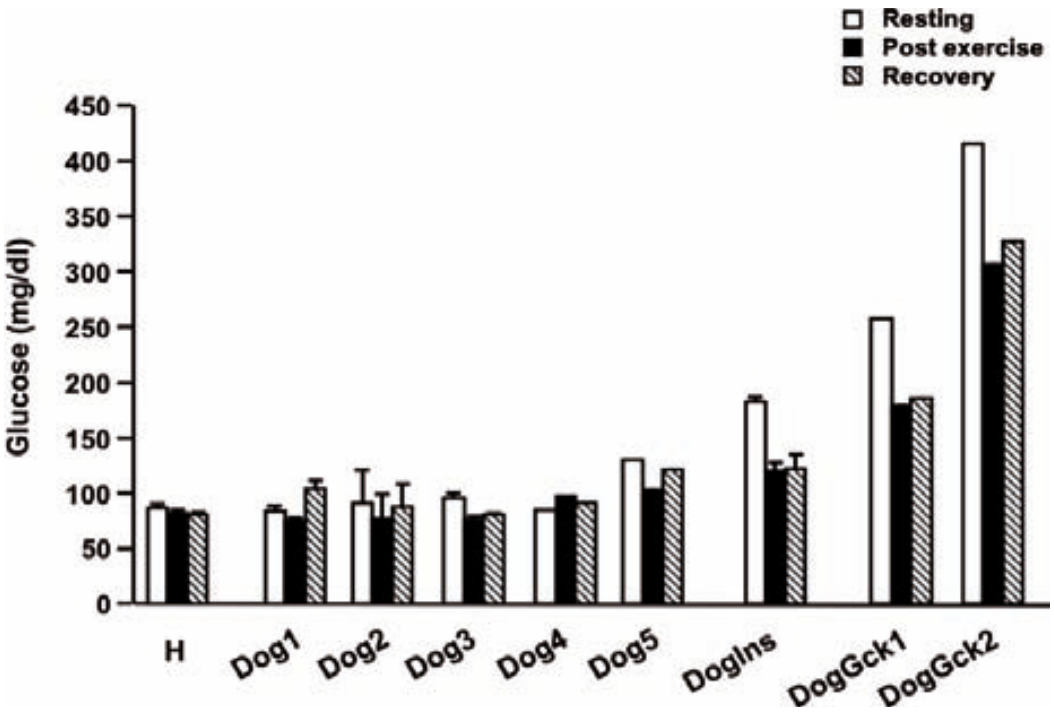

Figure 24. Effect of exercise on glycemia. Fasted dogs were challenged by 37 minutes of progressive moderate exercise to assess the risk of development of hypoglycemia. Blood glucose was measured in 24-hour fasted dogs before, immediately after exercise and during post-exercise recovery, a time equal to the period of exercise.

Thus, Gck expression in skeletal muscle in addition to insulin expression was safe during exercise and was in agreement with the low, but sufficient levels of fasting insulin levels in AAV-Ins and AAV1-Ins+Gck treated dogs and with the fact that Gck is inactive when glycemia is low, due to its high Km for the glucose (Printz et al., 1993b).

7. EVALUATION OF THE APPEARANCE OF SECONDARY COMPLICATIONS

Development of diabetic secondary complications are common in diabetic dogs and include blindness and anterior uveitis resulting from cataract formation, diabetic retinopathy, chronic pancreatitis, recurring infections, hepatic lipidosis, hypoglycemia and ketoacidosis (Nelson, 2010).

All the dogs in the study were periodically evaluated for the possible development of secondary complications at the Veterinary Clinical Hospital at UAB throughout the study. According to their elevated fructosamine levels and poor glycemic control, DogsDb1-3, DogsGck1 and DogGck2 developed diabetic cataracts. However, dogs treated with AAV1-Ins and AAV1-Ins+Gck never developed secondary complications according to their fructosamine levels within the range of excellently controlled diabetes (Table 3).

Table 3. Evaluation of secondary complications in insulin and AAV treated dogs.

Complication (Frequency in diabetic companion dogs)		Dog
Ocular complications	*Cataract* (Common)	Detected in DogDb1-3, DogGck1 and DogGck2 soon after diabetes induction with worsening over follow up period. Not detected in DogIns, Dog1, Dog2, Dog3 and Dog5.
	Uveitis (Common, secondary to cataract)	Not detected
	Retinopathy (Uncommon)	Not detected
Urinary tract infection	(Common)	Not detected
Nephropathy	*Azotemia* (Uncommon)	Not detected in DogDb1-3, DogIns, Dog1, Dog2, Dog4, Dog5, DogDb, DogGck1 and DogGck2. Drug-induced moderate azotemia in Dog3*.
	Proteinuria (Uncommon)	Not detected
Clinical Peripheral Neuropathy	*Weakness, knuckling, abnormal gait, muscle atrophy, depressed limb reflexes, deficits in postural reaction testing* (Uncommon)	Not detected

This table summarizes secondary complications described in companion diabetic dogs (Nelson, 2010). *Dog3 showed moderate azotemia immediately after STZ+Alloxan administration, compatible with a toxic effect of these drugs as previously described (Rerup, 1970). No proteinuria or urinary tract infections were detected in Dog3 for the duration of the study.

Thus, these results suggested that the metabolic control in AAV1-Ins and AAV1-Ins+Gck treated dogs was good enough to prevent the development of secondary complications.

V. DISCUSSION

Since the 1922 breakthrough discovery of Banting and Best, who corrected hyperglycemia in dogs using pancreatic extracts, exogenous insulin administration has been the mainstay of diabetes therapy. Alternative therapies have been studied, but thus far only a handful of approaches, mainly involving allo- or xeno-transplantation of pancreatic islets, have reached clinical application (Robertson, 2010).

Here we propose a novel approach to control hyperglycemia, through genetic engineering of a “glucose sensor” in skeletal muscle using AAV vectors that has permitted long-term, clinically meaningful regulation of glycemia in a large animal model of diabetes. In our approach, insulin is supplied at constitutive basal levels in a continuous manner with no peaks. These levels appear to be low enough to allow GLUT 4 translocation (Wasserman et al., 2011) to the muscle membrane, but not too much to cause hypoglycemia. Due to the constant levels of circulating insulin, glucagon levels will not rise when glycemia drops, so liver glucose output will not be activated, emphasising that a tight regulation of skeletal muscle glucose uptake is required to avoid hypoglycemia. In parallel, due to its high *Km* for glucose (Printz et al., 1993b), Gck expressed in the muscle will be active and phosphorylate glucose only when glycemia is high, but will remain inactive when glycemia drops, maintaining circulating glucose levels within an adequate range and thus, preventing hipoglycemia if the constant insulin expression is kept at a low level.

Currently, the goal of normalization of glycemia is pursued through intensive insulin therapy, which can delay the onset and slow the progression of secondary complications of diabetes (Diabetes Control and Complications Trial, 1993). However, this treatment is not suitable for all diabetic patients because of its high risk of hypoglycemia secondary to excessive insulin dosage (Diabetes Control and Complications Trial, 1993). On the other hand, islet or pancreas transplantation therapy is limited by the little amount of material available from cadaveric donors, the need of life-long immunosuppression and the rapid loss-of-function of the grafts (median insulin independence is 15 months) (Robertson, 2010; Ryan

et al., 2005; Van Belle et al., 2008; Correa-Giannella et al., 2009). Also, research into autologous stem cells to generate new β-cells has, on one hand, the advantage that they can be transplanted without the need of the same immunosuppressive drugs used for allotransplantation, but still have the drawback of the endogenous immune response against β-cells (Collombat et al., 2009; Fujita et al., 2004; Kroon et al., 2008; Minami et al., 2005; Okuno et al., 2007; Thorel et al., 2010; Yu et al., 2009). Our approach circumvents a number of the challenges of current and experimental diabetes treatments because no immunosuppresion appears to be necessary to achieve long-term (>10 years) expression in humans (Brantly et al., 2009; Buchlis et al., 2012) and AAV vector manufacturing is robust and unlimited (Ayuso et al., 2010).

Our approach of using two different transgenes is supported by several findings. Insulin alone, either provided as exogenous therapy or expressed in muscle by an AAV vector, did induce glucose uptake, however it did not guarantee a tight control of glycemia, especially after a glucose challenge. Expression of Gck alone did not correct hyperglycemia either, since diabetic dogs treated with high doses of AAV1-ohGck were hyperglycemic in fasting conditions. However, expression of Gck in skeletal muscle results in a greater sensitivity to insulin, either when insulin is exogenously administered or when it is expressed at low, safe levels from an AAV vector. Indeed, when an oral glucose tolerance test were performed at 1.75 g/kg, as recommended by the American Diabetes Association (ADA) to perform this test in humans (American Diabetes Association, 2011), glucose disposal was nearly normalized in all AAV1-Ins+Gck treated dogs and the glycemia at 2 hours after glucose load was below 200mg/dl of glucose disposal compared with diabetic curves, a profile considered no diabetic according to ADA guidelines (American Diabetes Association, 2011). These results demonstrated the synergistic role of Ins and Gck in the control of glycemia because neither Ins alone nor Gck alone can counteract hyperglycemia after a glucose load.

We next focused of the safety and long-term effects of this novel treatment. Follow-up of treated dogs for more than 4 years suggests that muscle expression of Ins and Gck is well tolerated over a prolonged period of time. Studies confirmed the safety of the approach even under marked physical exertion, when high levels of glucose consumption increase the risk of hypoglycemic episodes. Exercise-associated problems with glycemia is quite a common situation in diabetic patients since insulin supply is not continuous due to the fluctuations in insulin concentration associated with the release of the subcutaneously injected exogenous insulin. In contrast, in AAV-treated dogs, insulin is supplied at constitutive basal levels in a continuous manner with no peaks. These levels appear to be low enough to allow GLUT 4 translocation (Wasserman et al., 2011) to the muscle membrane, but not too much to cause hypoglycemia.

The use of a large animal model with a long lifespan allowed us to follow animals for early indicators of secondary complications. The absence of clinical findings such as cataracts or urinary tract infection, and the normalization of biomarkers such as glycosylated proteins (fructosamine), suggest that the glycemic control achieved by Ins and Gck gene transfer is good enough to prevent the development of diabetes secondary complications. Serum fructosamine levels represents the average blood glucose level over the preceding 2-3 weeks, not being affected their levels by acute increases in the blood glucose concentration (Nelson, 2010). Thus, fructosamine levels are used in veterinary medicine to assist in the diagnosis and monitoring of insulin treatment (Jensen, 1992; Marca et al., 2000; Nelson, 2010; Reusch et al., 1993; Thoresen et al., 1997). In AAV1-Ins and AAV1-Ins+Gck treated dogs, fructosamine levels were always within the range of excellently controlled diabetes throughout the study (Nelson, 2010).

Moreover, AAV1-Ins and AAV1-Ins+Gck treated dogs present with normal muscle morphology and normal glycogen accumulation, indicating that long-term expression of Ins and Gck from skeletal muscle did not induce muscle pathology.

Altogether, these results indicate that normalization of glycemia and prevention of secondary complications with a one-time intervention could result in a substantial improvement in patients' quality of life, particularly in populations with difficulties in diabetes management, such as patients that suffer so-called brittle diabetes characterized by a severe instability of glycemia with frequent hypoglycemic and ketoacidosic episodes (Bertuzzi et al., 2007; Vantyghem et al., 2006).

Skeletal muscle is an ideal tissue for gene therapy approaches because it is easily accessible by non-invasive procedures, it can efficiently secrete proteins into the bloodstream, the expression is stable for many years after gene delivery and transduction efficiency is not limited by the presence of pre-existing neutralizing antibodies, a key aspect given the relatively high prevalence of anti-AAV antibodies in the general population (Boutin et al., 2010; Buchlis et al., 2012; Kay, 2011; Manno et al., 2003; Mingozzi et al., 2011). Specially, is also ideal for type 1 diabetes gene therapy approaches since it is the most important site of glucose removal from blood, accounting for about 60 to 70% of glucose disposal after a meal (DeFronzo et al., 1981). AAV vectors are becoming the vector of choice for such *in vivo* gene therapy approaches due to their excellent safety and efficacy profiles (Mingozzi et al., 2011). For transducing the skeletal muscle, AAV1 are amongst the best serotypes (Wu et al., 2006). High levels of proteins, including insulin and proinsulin, can be secreted into the bloodstream after AAV1 intramuscular administration to the skeletal muscle (Fernandez-Sanchez et al., 2012; Mas et al., 2006; Riviere et al., 2006). Here we demonstrated skeletal muscle transgene expression in DogIns and Dog1 nine months after treatment AAV treatment and 2 years after treatment in Dog3. This was in agreement with human C-peptide detection throughout the study in all AAV1-Ins and AAV1-Ins+Gck treated dogs. Previous studies suggested that about 90% of the insulin produced *in vitro* by C_2C_{12} differentiated myoblast cells was processed by furin endoproteases and, released to the media together with the other part in the unprocessed proinsulin form (Gros et al., 1999). Moreover, previously in our laboratory it was demonstrated that human insulin expressed in

the skeletal muscle in a transgenic mice resulted in detectable circulating human C-peptide (Riu et al., 2002).

In addition, skeletal muscle AAV intramuscular injection leads to minimal systemic biodistribution (Arruda et al., 2001). Indeed, we observed in Dog3 that AAV-injected skeletal muscle necropsy samples contained a high number of vector genomes, whereas very low vector gene copy numbers were detected in most of peripheral tissues. These data correlate with previous findings using this serotype and route of administration, in which most of the detectable vector was expected to be found in the skeletal muscle (Leger et al., 2011; Toromanoff et al., 2008).

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In the present study, the selection of the dog as a large animal model of diabetes was based on the fact that the dog has had historically an important role in diabetes research and thus, there are many tools and biological references available. Indeed, the beneficial effects of insulin-containing extracts were first demonstrated in a dog (Banting et al., 1922). Moreover diabetes was first produced experimentally in a dog and that a role of the pancreas in the disease was thereby recognized (Engerman et al., 1982). Since then, diabetic dogs have been studied as both companion animals in veterinary medicine and as chemically-induced experimental models. The dog is an outbred animal with a long lifespan which allows the long-term assessment of treatment efficacy and safety of new therapeutic approaches (Gale, 2005). Furthermore, diabetic dogs commonly develop secondary complications resulting from diabetes or its treatment (Ettinger et al., 2010). Unfortunately, large animal models of autoimmune diabetes are not available and one possible limitation of the results presented here is that the dog model of diabetes used in this study does not mimic the immunological state of type 1 diabetic patients. However, while future studies in autoimmune -rodent- models of diabetes are warranted, studies in mice (Velazquez et al., 2009), dogs (Haurigot et al., 2010) and humans (Mendell et al., 2009) would suggest that targeting muscle with AAV vectors may at least partially escape immune recognition. This may be the

result of lower levels of MHC class I presentation in this tissue, or the result of the induction of apoptosis of reactive T cells (Mendell et al., 2009; Velazquez et al., 2009). Thus, we hypothesize that skeletal muscle Ins expression will not be sufficient to trigger an autoimmune attack against Ins-expressing cells because many other β-cell antigens might be required to break the immune tolerance.

In the clinical translation of bench results, the scale up to a large animal model represents perhaps one of the most critical steps. Indeed, in gene therapy field most proof-of-concept studies in mice has failed when they have been scaled-up to large animal models. Conversely, successfully studies in large animal models have been predictive of a positive outcome in clinical trials, as it has been nicely demonstrated in hemophilia B (Manno et al., 2006; Mount et al., 2002; Nathwani et al., 2007; Nathwani et al., 2011) and Leber's Congenital Amaurosis (Acland et al., 2005; Maguire et al., 2008). It should be noted that, for instance in the field of oncology, veterinary clinical trials in companion animals are often performed to test novel therapeutics before moving to the clinic (Gordon et al., 2009). These veterinary clinical trials in outbred companion animals, thus, in a very variable context, represents a much closer scenario that the one that will be found in a clinical trial, that those found in studies with inbred mice. The future clinical trial in diabetic companion dogs will greatly help to determine the feasibility and safety and efficacy profile of our approach in a possible future clinical trial in humans. Furthermore, the European Medicines Agency has recently granted a market authorization for the first gene therapy product in Europe consisting of an AAV1 vector for treating lipoprotein lipase deficiency, which gives further support for the potential translation of our approach to the clinic.

In summary, this work is noteworthy for several reasons:

- It is the first study of long-term control of diabetes in a large animal model of the disease. This has never been achieved before with any of the emerging gene and cell therapies for diabetes.

- It is the first study describing a single therapeutic intervention (intramuscular injection of AAV vectors) to achieve long-term (>4 years) maintenance of normoglycemia after development of diabetes.
- In addition to the primary endpoint of normoglycemia, the claim of efficacy is further bolstered by findings from long-term observation of the dogs, including normal levels of glycosylated proteins, and absence of secondary complications.
- It provides insights into the synergistic action of insulin and glucokinase in achieving tight control of glycemia.
- Finally, we provide abundant data demonstrating the long-term safety of the AAV-mediated gene therapy in this large animal model of diabetes.

The long-term safety and efficacy results presented in this study will be key data for supporting a first-in-human study for patients with diabetes.

VI. CONCLUSIONS

1. AAV1-Ins and AAV1-Ins+Gck treatment in diabetic dogs results in better glycemic control than exogenous insulin treatment.

2. AAV1-Ins+Gck treatment in diabetic dogs results in normalization of fasting glycemia, fasting insulinemia and body weight maintenance for more than 4 years.

3. Gene transfer with AAV1-Ins+Gck improve glucose disposal after an oral load of 3 g/kg and normalize glucose disposal after an oral load of 1.75 g/kg.

4. AAV1-Ins and AAV1-Ins+Gck-treated dogs show fructosamine levels within the range of excellently controlled diabetes throughout the study. In agreement, these dogs do not develop secondary complications.

5. Neither Ins alone nor Gck alone can counteract hyperglycemia after a glucose load. Thus, Ins and Gck act synergistically to achieve a tight glycemic control.

6. Proinsulin is expressed, correctly processed to mature insulin and released into the circulation from skeletal muscle in diabetic dogs.

7. After intramuscular administration of AAV1 vectors, most of the vector is found in the skeletal muscle.

8. AAV1-Ins and AAV1-Ins+Gck treated dogs present normal muscle morphology and normal glycogen accumulation, indicating that long-term skeletal muscle expression of Ins and Gck do not induce muscle pathology.

9. AAV1-Ins and AAV1-Ins+Gck treated dogs do not develop hypoglycemia or hyperglycemia in response to exercise.

10. Therefore, this study represent the first demonstration of long-term correction of diabetes in a large animal model using gene transfer, and lay the foundation for the clinical translation of this approach to veterinary medicine. Additional safety and efficacy studies will help evaluate the feasibility of the approach in humans.

VII. MATERIAL AND METHODS

1. MATERIALS

1.1. Bacteria stock and plasmidic vectors

The bacterial stock used to carry the different plasmidic vectors has been *E.Coli XL2Blue* (Stratagene, Santa Clara, California, USA). The growth of these cells has been done at 37ºC in LB medium (Miler's Luria Broth, Conda, Madrid, Spain), containing 50 µg/ml of Ampicilin.

1.2. Animals

1.2.1. Mice

8-week old mice C57Bl6SJL were used and they were purchased from Harlan (Indianapolis, Indiana, USA). Mice were kept in a specific pathogen-free facility at the Center of Animal Biotechnology and Gene Therapy (SER-CBATEG) under a 12-h light/dark cycle (lights on at 8:00 A.M.) with controlled temperature and has free access to water and standard diet (Teklad Global, Harlan Teklad, Madison, Wisconsin, USA). At the end of the experiment mice were sacrificed by means of inhalation of anaesthetics Isofluorane (IsoFlo ®; Abbott Animal Health, Abbott Park, Illinois, USA) and decapitated. Blood and tissues of interest were excised and kept at -80ºC.

1.2.2. Dogs

Male Beagle dogs (6-12 months-old) were purchased from Isoquimen (Sant Feliu de Codines, Spain) and housed at Servei de Granjes i Camps Experimentals of the Universitat Autonoma de Barcelona (UAB). Animals were fed individually once daily at 9:00 AM with 30 g/kg body weight standard dry food (Elite Nutrition). The dog studies were performed with the collaboration of Dr. Fèlix García and Dra. Anna Andaluz of the Department of Medicine and

Animal Surgery at the Veterinary School of UAB that were involved in all the dogs experimental procedures, including viral vectors injections, biopsies and animal euthanasia. Dr. Rafael Ruiz de Gopegui of the Department of Internal Medicine at the Veterinary School of UAB supervised regularly the health of the dogs during all the experiments. To this end dogs undergone previously to began the experiment a complete clinical exam including thorax radiography, ecografic evaluation, electrocardiogram, eye examination, urianalysis, and haematological and biochemical analysis. Regularly during the experiment dogs underwent physical examinations, eye examinations and urianalysis. Haematological and biochemical analysis were performed monthly. At the end of the experiment, when required, dogs were euthanized with an intravenous solution of sodium pentobarbital.

All the experimental procedures were approved by the Ethics Committee in Animal and Human Experimentation of the UAB.

1.3. Probes

Human insulin probe came from a 0,4kb EcoRI-EcoRI digestion from the human Ins cDNA with an endoprotease furin cleavage signal (hIns) (given by Dr.W.Rutter) cloned in a pGG2/CMV vector (pGG2/CMV-hIns). Rat glucokinase probe come from a 2,3kb EcoRI-EcoRI digestion from the rat Gck cDNA (rGck) (given by Dr.P.Iynedjian, Genève University, Genève, Switzerland) cloned in a pGG2/CMV vector (pGG2/CMV-rGck).

1.4. Plasmids and viral vectors preps

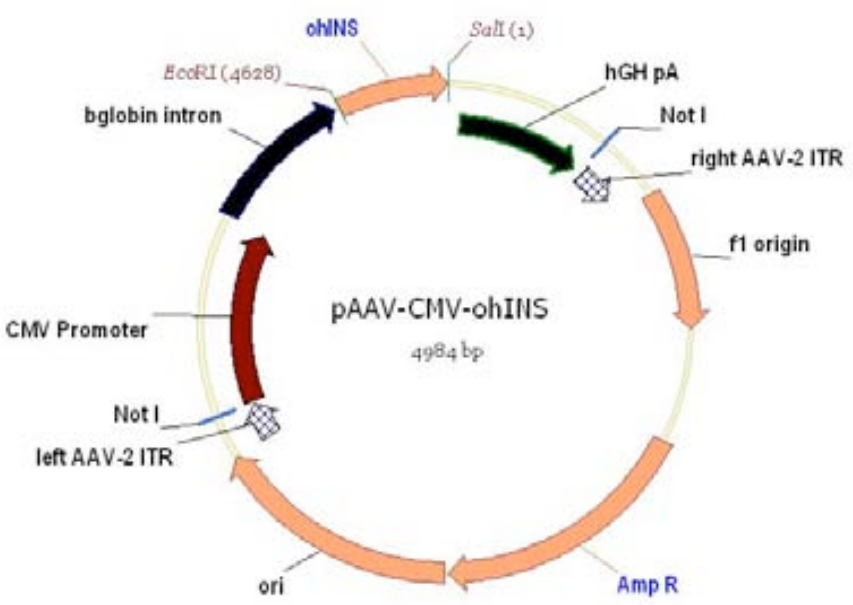
pGG2/CMV-hIns and pGG2/CMV-rGck plasmids were previously cloned and obtained from CBATEG. From these two plasmids, AAV1/CMV-hINS and AAV1/CMV-rGck were produced at the Center for Cellular and Molecular Therapeutics at the Children's Hospital of Philadelphia.

hIns and human Gck (hGck) genes were optimized (obtaining optimized hIns (ohIns) and optimized hGck (ohGck)) by Life technologies (San Diego, California, USA) with a GeneOptimizer® software that optimizes sequences for maximum transcription and translation. GeneOptimizer® sequence processing includes the following: a) eliminates cryptic splice sites and RNA destabilizing sequence elements for increased RNA stability, b) adds RNA stabilizing sequence elements, c) codon optimize the sequences and adapts the G/C content, d) intron removal and e) avoid stable RNA secondary structures (Fath et al., 2011). The codon usage was adapted to maximize translation. In addition, regions of very high (>80%) or very low (<30%) GC content were avoided when possible. The mutated gene constructs obtained showed CAI (codon adaptation index) of 0.96 what means high and stable expression rates. GC-content adjustment made by the process prolongs mRNA half-life of the optized constuct (Fath et al., 2011).

pMA-TohINS, pMA-TohGck plasmids were sent to us a from the company and the inserts were cloned in a pAAV-MCS receptor plasmid (Agilent technologies (Santa Clara, California, USA)). The genes where cloned under the control of a cytomegalovirus promoter (CMV) and the first intron of the human □-globin gene. Also the final constructs included a hGH polyA tail. The cloning was performed digesting pMA-TohINS, pMA-TohGck plasmids with EcoRI/SalI restriction enzymes (Fermentas, St.Leon-Rot, Germany) to obtain pAAV/CMV-ohINS (Figure 1A) and pAAV/CMV-ohGck (Figure 1B) plasmids. From these two plasmids AAV1/CMV-ohIns and AAV1/CMV-ohGck were produced. AAV1/CMV-ohINS was

produced at the Center for Cellular and Molecular Therapeutics at the Children's Hospital of Philadelphia and AAV1/CMV-ohGck at CBATEG. Also, from pAAV-MCS plasmid, AAV1/CMV-null was produced at CBATEG.

A



B

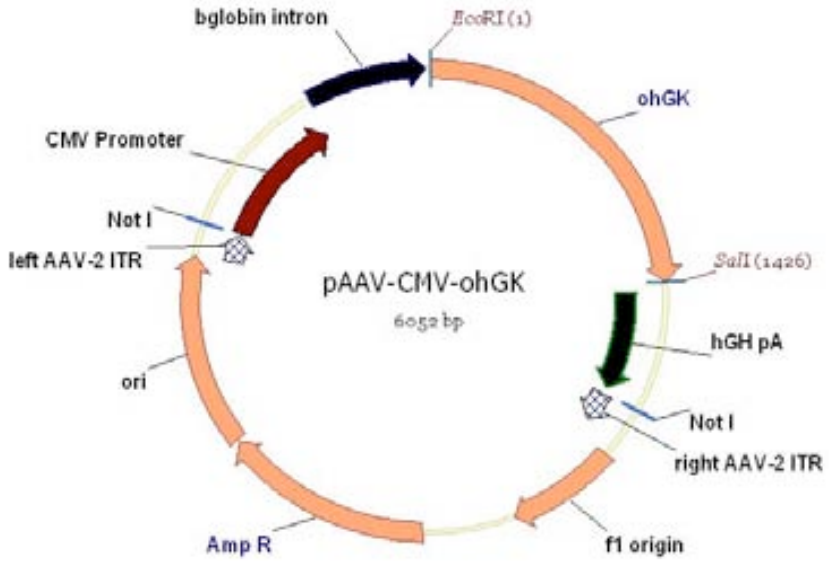


Figure 1. pAAV/CMV-ohINS (A) and pAAV/CMV-ohGck (B) plasmids.

1.5. Reagents

The molecular biology reagents used in this work were purchased in:

Life Technologies (San Diego, California, USA), Roche (Indianapolis, Indiana, USA), Bio-Rad (Hercules, CA, USA), Sigma (St.Louis, Missouri, USA), Promega (Madison, Wisconsin, USA), Qiagen (Hilden, Germany), Fermentas (St. Leon-Rot, Germany), Epicentre Biotechnologies (Madison, Wisconsin, USA), MP Biomedicals (Irvine, California, USA), New England Biolabs, (Ipswich, Massachusetts, USA), Agilent Technologies (Santa Clara, California, USA), Pharmacia-LKB (Uppsala, Sweden), Millipore (Billerica, Massachusetts, USA), Merck (New Jersey, USA), Dako (Glostrup, Denmark), Vector (Burlingame, California, USA), GE Healthcare (Little Chalfont, United Kingdom).

2. METHODS

2.1. Obtention of the DNA

2.1.1. Plasmidic DNA obtention

For the obtention of little amounts of plasmidic DNA, minipreparations were performed under the protocol of alkaline lysis originally described by Birnboim and colleagues (Birnboin and Doly, 1979). With this method 3-4 μg/ml of plasmid are obtained, that are mostly circular, covalently closed (*supercoiled*), and sensitive to enzymatic manipulations without the need of more purifications.

To obtain higher amounts of plasmid DNA required for cloning or AAV production, the PureYeld™ Plasmid MaxiPrep System from Promega Corporation (Madison, Wisconsin, USA) or the EndoFree Plasmid Mega Kit from Qiagen (Hilden, Germany) were used.

2.2. Enzimatic manipulation of DNA

A restriction enzyme is an enzyme that cuts double-stranded DNA at specific recognition nucleotide sequences known as restriction sites. Such enzymes, found in bacteria and *archaea,* are thought to have evolved to provide a defense mechanism against invading viruses. Inside a bacterial host, the restriction enzymes selectively cut up foreign DNA in a process called restriction, host DNA is methylated by a modification enzyme (a methylase) to protect it from the restriction enzyme's activity. Collectively, these two processes form the restriction modification system. To cut the DNA, a restriction enzyme makes two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix.

Each restriction enzyme requires specific reaction conditions of ionic strength, pH and temperature. A battery of 3 to 4 different buffers will handle a large number of available enzymes, although there are few that require a unique buffer environment. In all cases, a major function of the buffer is to maintain pH of the reaction (usually at 8.0). Most restriction enzymes cut best at 37ºC, but there are many exceptions.

By definition, 1 unit of restriction enzyme will completely digest 1 ug of substrate DNA in one hour. Usually the concentrations of the restriction enzymes are 10 U of enzyme per each ul of enzyme. However usually is often followed a protocol where 5-10 fold overdigestion is recommended to overcome variability in DNA source, quantity and purity.

The restriction enzyme is supplied in 50% glycerol and the restriction enzyme should not exceed 10% of the total reaction volume. This is because is important to keep glycerol concentration at less than 5% of the total reaction volume to prevent star activity. Star activity that is a relaxation or alteration of the specificity of restriction enzyme mediated cleavage of DNA that can occur under reaction conditions that differ significantly from those optimum for the enzyme. The result is typically cleavage at non-canonical recognition site, or sometimes complete loss of specificity. This can be due to high glycerol concentration (> 5% v/v), high concentration of enzyme/µg of DNA ratio, the use of a non optimal buffer, prolonged reaction time, presence of organic solvents and the substitution of Mg^{2+} with other divalent cations (Mn^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}).

The incubation time is depending on the amount of DNA that has to be digested and the amount of the restriction enzyme used in the reaction, but prolonged reaction time is not recommended (typically more than 3 hours).

The restriction products were analyzed or processed in an agarose gel.

2.3. Isolation and purification of DNA fragments

2.3.1. Agarose gel electrophoresis

Agarose gel electrophoresis was the method used for the separation of DNA fragments. This technique allows separate and identificate DNA fragments ranging from 50 base pair to several megabases (millions of bases). The distance between DNA bands of a given length is determined by the percent agarose in the gel. One per cent agarose gels were used to resolve DNA fragments between 0,7 up to 10 kilobases.

The DNA fragments visualization was achieved adding to the gel low concentrations ethidium bromide (0.5 □g/ml) a fluorescent dye, which inserts itself into the spaces between the base pairs of the double helix. The DNA was visualized with low wavelength (310 nm) ultraviolet light using the transilluminator and camera system (Syngene, Cambridge, United Kingdom). Ethidium Bromide is a sensitive, easy stain for DNA. It yields low background and a detection limit of 1-5 ng /band. Ethidium staining is strongly enhanced by the double stranded structure of native DNA. Staining of denatured, ssDNA or RNA is relatively insensitive, requiring some 10 fold more nucleic acid for equivalent detection.

Agarose gels were done dissolving agarose in 1X TAE (Trisacetate pH 8.3, 40mM and EDTA 1mM). To determine the molecular weight of the DNA fragments it was used the 1Kb DNA Ladder from Life Technologies (San Diego, California, USA). Samples were loaded mixing them with a loading buffer that contained a dye to assess how the gel is running and to render the samples denser than the running buffer and remain them in the well. The loading dye buffer used was a 6X Loading Dye from (Fermentas St. Leon-Rot, Germany) composed of Glycerol 50%, EDTA 100mM, SDS 1% and bromophenol blue 0.1%.

After, the gel was run at a maximum of 100 V for the required time to have the desired

bands well separated.

2.3.2. DNA fragments purification

DNA was run in the agarose gel and the desired DNA fragments were cut from the gel and subsequently purified to extract the DNA.

The kit used to extract and purify the DNA was the Geneclean® Turbo Kit (MP Biomedicals, California, USA). This method consists in three steps that are bind, wash, and elute. First of all the gel fragment was dissolved in a saturated NaCI solution, that allows DNA to be selectively trapped in a silica-embedded membrane in the next step. Then, the DNA was washed and subsequently was eluted using a low ionic strength solution.

2.4. Construction of recombinant DNA molecules

2.4.1. Dephosphorilation of DNA fragments

Self-ligation of DNA can be avoided by enzymatic removal of phosphate residues from DNA's 5' termini with phosphatases. The Shrimp Alkaline Phosphatase (Promega, Madison, Wisconsin, USA) was used and dephosphorylation was performed using manufacturer's instructions. DNA was dephosphorilated for 30 min at 37ºC. Upon completion, the enzyme was inactivated by heating the mix to 65ºC for 15 min.

2.4.2. Fragment ligation

Joining linear DNA fragments together with covalent bonds is called ligation. More specifically, DNA ligation involves creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another.

The enzyme used to ligate DNA fragments is T4 DNA ligase (New England Biolabs, Ipswich, Massachusetts, USA), which originates from the T4 bacteriophage. This enzyme will ligate DNA fragments having overhanging, cohesive ends that are annealed together. T4 DNA ligase will also ligate fragments with blunt ends, although higher concentrations of the enzyme are usually required for this purpose. Reactions were carried out in the presence of ligation buffer with ATP at room temperature overnight.

2.4.3. Transformation of competent E.Coli XL2Blue.

Transformation consists on introducing a foreign plasmid into a bacteria and to use that bacteria to amplify the plasmid in order to make large quantities of it.

Electroporation is a transformation method that consists in provoking a significant increase in the electrical conductivity and permeability of the cell or bacteria plasma membrane caused by an externally applied electrical field.

Plasmid DNA was introduced into competent *E.Coli XL2Blue* by transformation. In these strain of *E.Coli* the electroporation is the best method of transformation. 40 µl of competent cells ($2X10^{10}$cells/ml) were thawed on ice and 1 µl of DNA was added directly to the cells. Cells and DNA were mixed and incubated on ice for 5 min. After that, cells were electroporated at 2500 V with an electroporator from Bio-Rad Laboratories (Hercules, California, USA). 200 µl of LB were added and cells were plated in LB plates with the appropriate antibiotic and incubated at 37ºC overnight. Only the cells that have acquired the plasmid will grow in the plate. These cells will form colonies that can be isolated. From this isolated colonies DNA was extracted using the miniprep method and using restriction enzymes and a subsequent run of the digested DNA in an agarose gel, it was checked if the plasmid of this colony is the one with the expected ligation product.

2.5. RNA preparation and analysis

2.5.1. RNA extraction

Total RNA extraction was performed in tissues that were rapidly removed after sacrificing the animal or from dog's biopsy and quickly frozen in liquid nitrogen.

After, the frozen tissues were homogenized in 1 ml of TripureTM Isolation Reagent (Roche Diagnostics Corp., Indianapolis, USA) with a Polytron® type tissue homogenizer.

A RNA extraction, using a phenol-chloroform extraction method and a guanidine thiocyanate as a ribonuclease inhibitor, were performed. The RNA fraction was extracted by addition of 0.2 ml chloroform/ml of Tripure and further purified using adsorption columns for RNA purification RNAEasy Mini Kit (Qiagen GmbH, Hilden, Germany), following the manufacture's instructions for RNA clean up procedure. All samples were treated on-column with DNAse I (RNAse-Free DNase Set for on-column sample treatment from Qiagen GmbH (Hilden, Germany)) and, after rising with the buffer provided by the manufacturer, eluted from the column with 30 □l of RNAse-free distilled water. Finally, the concentration of RNA in the different samples was determined by measurement of the absorbance at 260 nm using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.5.2 Gene expression analysis by Northern Blot

Total RNA from quadriceps was extracted from biopsy or necropsy samples of the dogs. All the solutions and buffers were prepared in RNAse-free conditions to avoid enzymatic degradation of the samples. Fifteen micrograms of total RNA were electrophoresed in a 2.2M formaldehid 1% w/v agarose gel. At this concentration the

formaldehyde denatures the RNA secondary structures allowing the migration of the RNA to be proportional to the logarithm of its molecular weight. Samples were run for 4-5 hours under 70 V. The RNA was subsequently blotted to positively charged nylon membranes (Roche Diagnostics Corp., Indianapolis, USA).

After a minimum of 2 hours of blotting, the blot was irradiated with 120000 μJ of ultraviolet light during 25-50 seconds in a UV-Statalinker 1800 (Stratagene, La Jolla, California, USA) to immobilize the RNA in the nylon membrane by generation of covalent bindings.

Agarose gel: 1% w/v agarose; 10X MOPS; 2.2M formaldehyde; RNAse-free sterile distilled water.
Sterile electrophoresis buffer: 0.2M MOPS; 50mM sodium acetate; 10mM EDTA (pH 7 adjusted with 10M NaOH). Filtered with 0.22 nm filter.
RNA loading buffer: 2% v/v deionized formamide; MOPS/EDTA 10X; 6.75% v/v 37% formaldehyde; 5.4% v/v glycerol; RNase-free sterile distilled water and bromophenol blue as a dye. Filtered with 0.22 nm filter.

After the transfer the transcripts were detected by probing with radioactively labelled sequences corresponding to the cDNA of hINS or rGck, obtained as explained in 1.3.

First of all, the nylon membrane to which it has been blotted the RNA was pre-hibridated at 65 ºC during 1 hour in rotation with a 10% pre-hibridation solution.

To radioactively label a probe the commercial kit Ready to Go® (GE Healthcare (Little Chalfont, United Kingdom)) following the manufacture's instructions. First of all, 25 ng of probe were taken with MiliQ-Water to a final volum of 45 ul and boiled during 10 min. Following this, the probe was put 2 min on ice and, after this, it was dissolved in the

commercial lyophilized complex that contains a mix of oligonucleotides (dATP, dGTP and dTTP) and the *E.Coli* DNA polymerase klenow fragment. When dissolved, a 5 □l solution of [□32P]dCTP was added and incubated for 30 min at 37ºC. In this step, the complementary chain of the probe is radioactively tagged with a large amount of specific radioactivity ($1,8x10^9$ dpm/mg). Following this, the probe was boiled another time to denaturalize the double strain and obtain single labelled strains and, finally the denatured probe was added to the hibridation tube, which was left in rotation overnight at 65ºC.

Next day, washes were performed: a first step of two washes of 10 min with a low stringency solution (300mM NaCl; 30mM sodium citrate and 0.1% SDS) were performed followed by a second step consisting on one wash of 10 min with a high stringency solution (15mM NaCl; 1.5mM sodium citrate and 0.1% SDS). Afterwards, the membranes were exposed in an imaging plate within an imaging cassette (Fujifilm (Dusseldord, Germany)) for a minimum of 3 hours. After, the imaging plate was read in a Fluorescent Image Analyzer FLA-3000 (Fujifilm (Dusseldorf, Germany)) that is able to detect fluorescent and radioisotopic images. For a cleaner and higher resolution northern image, the membranes were exposed to an autoradiographic film (GE Healthcare (Little Chalfont, United Kingdom)) and developed between 1 and 7 days later depending on the potency of the radioactive signal previously observed in the imaging plate.

2.6. DNA preparation and analysis.

2.6.1. DNA extraction

Total DNA was isolated with MasterPureDNA Purification Kit from Epicentre Biotechnologies (Madison, Wisconsin, USA) following the manufacture's instructions. First of all 1 □l of proteinase K (that rapidly inactivates nucleases that might otherwise degrade the DNA during purification) was diluted into 300 ml of Tissue and Cell Lysis Solution for each

sample (a piece between 1-5 mg in the case of dog muscle). The Tissue and Cell Lysis Solution containing the proteinase K was added to sample and incubated at 65°C for 15 min. After this, the samples were cooled to 37°C and 1 □l of 5 □g/□l RNase A was added to each sample. Subsequently samples were incubated at 37°C for 30 min. Then, the samples were placed on ice for 3-5 min and 175 □l MPC Protein Precipitation Reagent was added to make the proteins precipitate. Afterwards, the samples were centrifuged at 4°C for 10 min at ≥10,000g. To the recovered supernanant, isopropanol was added to make the DNA precipitate and the samples were centrifuged at 4°C for 10 min at ≥10,000g. Next, the pellet was washed twice with 70% ethanol. Finally, the DNA was resuspended in H_2O MiliQ. The concentration of DNA in the samples was determined by measurement of the absorbance at 260 nm using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.6.2. Viral genomes determination by quantitative PCR.

A real time quantitative PCR with a TaqMan probe was used for the determination of the viral genomes content. TaqMan probes are hydrolysis probes that are designed to increase the specificity of real-time PCR assays. The TaqMan probe principle relies on the 5´–3´ exonuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorophore-based detection.

Vector genome copy number was determined in 1 □l of H_2O MIliQ + DNA, containing 20 ng of genomic DNA with primers and probe specific for CMV promoter. A LightCycler® 480 Probes Master (Roche (Indianapolis, Indiana, USA)) was used following the manufacture's instructions. In a 96-well plate are placed for a 20 □l reaction:

- DNA 1 □l

- PCR Mix 10 □l
- Forward primer (10 □M) 1 □l
- Reverse primer (10 □M) 1 □l
- TaqMan primer 0.2 □l
- H_2O MIliQ 6,8 □l

The PCR reaction consisted in 45 cycles of 10 seconds at 95ºC and 30 seconds at 60ºC. Each sample was performed in triplicate

Using a standard curve used amplified also with the same primers in the same reaction, vg/20ng of genomic DNA were calculated. To calculate the number of vg/cell we have to divide the obtained value of vg/20 ng of genomic DNA per 3378,4, because it has been described that in 20 ng of DNA there are 3378,4 diploid cells.

The primers for TaqMan qPCR with and probe specific for CMV (cytomegalovirus) promoter for viral genomes determination were:

Name	**Primer Fw**	**Primer Rv**
CMV	CACCAATGGGCGTGGATAGC	GCAGTTGTTACGACATTTTGGAAA

Probe 5' – 3' ATTTCCAAGTCTCCACCC

Primers were purchased from Life technologies (San Diego, California, USA)

2.7. Glucokinase activity determination

To measure Gck activity in mice skeletal muscle, tissues biopsies were obtained from anaesthetized mice. Frozen gastrocnemius samples were homogenized in an ice-cold buffer (pH 7.4) containing 100 mM Gly-Gly, 200 mM ClK, 5 mM DTT and 65 mM Tris. Samples were then centrifuged to pellet insoluble material. The glucose phosphorylation capacity was assayed in the supernatants at 30°C in a buffer containing 50 mM Gly-Gly, 100 mM KCl, 2.5 mM DTT, glucose-6-phosphate dehydrogenase (1 U/ml), 0.5 mM NADP and 4.5 mM ATP-Mg. Gck activity was calculated as the difference between the glucose phosphorylation capacity at 100mM and 0.5mM glucose. Protein content was measured by Bradford assay (Pointe Scientific, USA) and Gck activity was expressed as mU/mg of protein.

2.8. Adenoassociated vectors production

2.8.1. AAV production and purification

Infectious AAV vector particles were generated in HEK293 cells cultured in roller bottles (RB), by co-transfecting each RB with 125 µg of the vector plasmid (containing the ITRs and the expression cassette) together with 125 µg of the rep/cap plasmid (expressing capsid proteins of the AAV particle and proteins necessary for virus replication) and 150 µg of the helper plasmid pWEAD expressing adenovirus helper functions by calcium phosphate coprecipitation (rep/cap and pWEAD plasmids were kindly provided by Dr.High, Children's Hospital of Philadelphia, USA). A total of 100 RB were used for each vector preparation. Three days after transfection, cells were harvested and centrifuged at 2500g for 10 min. Cell pellet was thoroughly reconstituted in TBS (50 mM TrisHCl, 150 mM NaCl, 2mM $MgCl_2$, pH 8.0). After 3 freeze/thaw cycles the lysate was centrifuged at 2500g for 30 min. Supernatant from this centrifugation was added to the medium and vector particles were precipitated by incubation with 8% of PEG 8000 (Sigma (St.Louis, Missouri, USA)) for 15 hours and pelleted at 2500g for 30 min. This pellet, now containing vectors from cells and medium, was thoroughly reconstituted in TBS (50mM TrisHCl, 150mM NaCl, 2mM $MgCl_2$, pH 8.0), treated

with benzonase (Merck (New Jersey, USA)) for 30 min at 37ºC and centrifuged at 10000g for 10 min. The supernatant was loaded into 37.5 ml ultra clear tubes (Beckman (Brea, California, USA)) containing 1.3-1.5 g/ml CsCl density step gradient, and centrifuged for 17 hours at 28000rpm in a SW28 rotor (Beckman (Brea, California, USA)). Viral bands were carefully collected using a 10 ml syringe and 18-gauge needle and transferred to a new 12.5 ml ultraclear tube, which was filled up with 1.379 g/ml CsCl solution to generate a continous gradient. Tubes were centrifuged at 38000 rpm in SW40Ti rotor (Beckman (Brea, California, USA)) for 48 hours. Finally, the band of full particles was collected and dialyzed in PBS using 10 KDa membrane (Slide-A-Lyzer Dialysis Products, (Thermo Fisher Scientific, Waltham, Massachusetts, USA)). And filtered with 0.45 µm Millipore filters. This PEG and CsCl-based purification protocol dramatically reduces empty AAV capsids and DNA and protein impurities from the viral stock, thus increasing AAV purity, which ultimately results in higher transduction *in vivo* (Ayuso et al., 2009).

2.8.2.Viral genomes quantification by quantitative PCR

Titers of vector genomes were determined by quantitative PCR assay following the protocol described for the AAV2 Reference Standard Material using linearized plasmid DNA as standard curve (Lock et al., 2010). Quantification of each vector lot was compared to a vector lot with known concentration to ensure the validity of results. Specifically, the reference lot used in this work was the AAV2 Reference Standard Material AAV2RSS (Lock et al., 2010).

To ensure that vector titer was not overestimated due to presence of remaining DNA plasmids in the lot, a DNAse treatment was performed before the quantification. Thus, 5 µl of each vector lot were added to 44.5 µl of DNAse buffer (13mM Tris, pH 7.5; 5mM $MgCl_2$) and 0.5 µl of DNase (10 U) and incubated for 30 min at 37 ºC.

Vector quantification was performed by qPCR, some with SyBrGreen and some with TaqMan.

PCR Mix used for SyBrGreen was LightCycler® 480 SYBR Green I Master (Roche (Indianapolis, Indiana, USA) and PCR Mix used for TaqMan was LightCycler® 480 Probe Master (Roche (Indianapolis, Indiana, USA))

SyBrGreen reaction:

- PCR Mix 10 µl
- Forward primer (10 □M) 5 µl
- Reverse primer (10 □M) 5 µl
- H_2O MiliQ 4 µl
- Diluted vector 5 µl

The PCR reaction consisted on 45 cycles of 15 seconds at 95ºC, 30 seconds at 58ºC and 30 seconds at 60ºC.

Taqman reaction:

- PCR Mix 10 µl
- Forward primer (10 □M) 1 µl
- Reverse primer (10 □M) 1 µl
- TaqMan probe 0,2 µl
- MiliQ H_2O 2,8 µl
- Diluted vector 5 µl

The PCR reaction consisted on 45 cycles of 10 seconds at 95ºC and 30 seconds at 60ºC.

The exact name of the prep vector, lots used as reference as well as the primers used for titration of the preps used in this work are indicated in the following table:

Prep	Reference lot	qPCR method	TaqMan probe	Primer Fw	Primer Rv
AAV1GFP (48)	AAV2RSS	SyBr Green	-	CAATAGGGACT TTCCATTGACG	AAGGTCATGT ACTGGGCATAA
AAV1hINS (KA41)	AAV2RSS	SyBr Green	-	CAATAGGGACT TTCCATTGACG	AAGGTCATGT ACTGGGCATAA
AAV1ohINS (KA564)	AAV2RSS	SyBr Green	-	CAATAGGGACT TTCCATTGACG	AAGGTCATGT ACTGGGCATAA
AAV1rGck (KA42)	AAV2RSS	SyBr Green	-	CAATAGGGACT TTCCATTGACG	AAGGTCATGT ACTGGGCATAA
AAV1ohGck (312)	AAV2RSS	TaqMan	CTGGCTGAC CGCCCAACGA	CAATTACGGGGTC ATTAGTTCATAGC	ATACGTAGATGTA CTGCCAAGTAGGA

2.8.3. Quantification of viral capsides by silver staining

Analysis of the viral preparations by protein electrophoresis and subsequent silver staining enables the quantification of the physical particles, which, since the PEG and CsCl method of purification yields vector lots with almost 100% full capsids, would provide another titer to be compared with the vector genome titer (see section 2.8.2). Furthermore, this method can also reveal if the preparation is contaminated with other non-viral proteins that might affect the transduction efficiency of the preparation. It must be kept in mind that quantification of physical particles is not dependant on the AAV serotype of the lot.

Briefly, appropriate volumes of the tested vector and of several dilutions of a reference vector lot were mixed with 2X Novex® Tris-Gly SDS Sample Buffer (Life Technologies, (San Diego, California, USA)) and 10X Novex® Tris-Gly Mini Gel (Life Technologies, (San Diego, California, USA)) and run at 125 V for 1.5-2 hours. The gel was

then stained using SilverXpress® Silver Staining Kit (Life Technologies, (San Diego, California, USA)), following manufacturer's instructions.

2.9. AAV vectors administration

2.9.1. In mice

For AAV1 injection to the skeletal muscle, mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) (Imalgene 500□, Merial, Barcelona, Spain) and xylacine (10 mg/kg) (Rompun□, Bayer, Leverkusen, Germany). Both back hindlimbs were shaved to easily inject the *tibialis anterior*, *gastrocnemius* and *quadriceps*. 30 □l of a solution that contains the required dose of the diluted vector in $Ca^{2+}Mg^{2+}$PBS (Life Technologies (San Diego, California, USA)) was injected using a 0.5 ml insulin syringes (B.Braun, Melsungen, Germany). After the injections, mice were returned to cage.

2.9.2. In dogs

In the AAV-treated dogs, vectors were delivered to a total of 12-25 sites in the *quadriceps* (with a 5-prong needle syringe) and in the *tibialis* (single point injections) of both hind limbs, with maximal vector dose/site of $<6x10^{11}$ vg (Figure 2).

First of all, a catheter was placed (Vasocan®, B. Braun, Sheffield, United Kingdom) in the cephalic vein of one of the forelegs to deliver the drugs. Then, the dog was pre-medicated with intravenously buprenorphine hydrochloride (Buprenex®, Merk Corporate, NJ, United Stated) at a dose of 0.01 mg/kg. Anaesthesia was induced with an intravenous injection of Propofol (Propovet®, B. Brau, Sheffield, United Kingdom) at a dose of 4 mg/kg and also Diazepam (Valium®, F.Hoffmann-La Roche, Basel, Switzerland) was administered at a dose of 0.5 mg/kg. Next, the animal was intubated and the anaesthesia was maintained

with the inhalation of vapour of 2% isoflurane with oxygen (150 ml/kg/min) in a semi-open breathing system Bain. Once the dog was anesthetized the injection area was shaved.

For the injections in the *quadriceps* the viral vectors were diluted in volumes of 2.5 ml of $Ca^{2+}Mg^{2+}$PBS until the total amount of the required dose was achieved and the injections were performed with a 5-prong needle syringe Meso-relle® (Biotekne SRL, Bologna, Italy) with a volum of 0.5 ml per injection site (Figure 2). For the injections in the *tibialis*, the viral vectors were diluted in volumes of 1 ml of $Ca^{2+}Mg^{2+}$PBS until the total amount of the required dose was achieved and the injections were performed using a 1 ml insulin syringes (B.Braun, Melsungen, Germany).

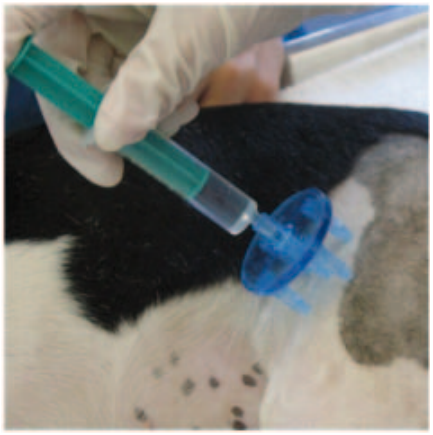


Figure 2. A 5-prong needle syringe was used as the vector administration device.

2.10. Diabetes induction

2.10.1. Diabetes induction in mice

To induce diabetes, mice aged 8 weeks were given, on 5 consecutive days, an intraperitoneal injection of STZ (Sigma (St.Louis, Missouri, USA)) at a dose of 45 mg/kg body weight dissolved in 0.1 mol/l citrate buffer (pH 4.5) immediately before administration. Diabetes was assessed by measuring fed blood glucose levels with a Glucometer Elite (Bayer, Leverkusen, Germany) every week after the last dose of STZ.

2.10.2. Diabetes induction in dogs

For the induction of diabetes the method of Anderson et al was used (Anderson et al., 1993). This method combines STZ and alloxan in order to use doses below the diabetogenic dose required for each chemical if used alone and reduce the toxicity that each drug used individually would produce in the dog. Chemically induced diabetes with STZ and alloxan at high doses can be fatally toxic, as a consequence of a possible pancreatitis and for the possible damage to other organs like the liver, but specially the kidneys. Also is important to be aware about the possible severe hypoglycemia due to the massive β-cell destruction that follows STZ and alloxan injection.

Dog weight and age must be taken into consideration. Older dogs were generally given a larger dose due to size, but correspondingly to a higher dose were more prone to lethal side effects within the first week, so the use of young and not very heavy dogs improve the outcome of the diabetes induction (Anderson et al., 1993).

Dogs are fasted for a minimum of 12-14 hours before drug administration to ensure basal blood glucose and avoid potential problems with vomiting. Catheters in the cephalic

vein in dogs' foreleg are placed for administration of the drugs. The dogs should not be fed at this stage. Water, however, should be given. STZ and alloxan must be prepared immediately before administration as they are unstable once in solution. The doses used are 40 mg/kg for alloxan (Sigma, St.Louis, Missouri, USA) and 35 mg/kg of STZ (Sigma, St.Louis, Missouri, USA) prepared in 100mM trisodium citrate buffer, pH 4.5. The solutions are made to a concentration of 100mg/ml and sterilised through 0.22 mm filters.

The hypoglycemic episodes begin about 8 hours after drugs injection and can last for up 8 hours. During this time, blood glycemia should be measured every hour. To prevent hypoglycemic episodes, dogs must receive a continous infusion of saline solution at 5% of glucose. If a hypoglycaemic episode occurs, boluses of saline solution at 50% of glucose are given until hypoglycemia is counteracted. From 24 to 48 hours after diabetes induction, basal blood glucose level begin to rise over 250 mg/dl. Glycemic controls are important to ensure that the glycemia doesn't rise too high, because very high sustained hyperglycemia can eventually produce ketoacidosis. For this reason, once hyperglycemia is stablished and sustained, normally between 2 and 3 days after diabetes induction, exogenous insulin treatment must be initiated to control the hyperglycemia and halt body weight loss. After this period, when dogs are stable can be returned to the kennel and normally fed, though monitoring should be performed very close. Biochemical analyses are very important to be performed every week until one month after diabetes induction to check for possible drug toxicity. Is important to check for the evolution of the renal parameters creatinine, urea and phosphorous, for the pancreatic parameters amylase and lipase to detect a possible acute pancreatitis and for the AST and ALT liver parameters. Acute pancreatitis and renal damage requires urgent medical attention. Usually acute pancreatitis can be resolved in few days, but renal damage sometimes remain as a permanent damage or derives to a renal failure and to death.

2.11. Insulin treatment in dogs

Insulin glargine (Lantus®(Sanofi Aventis, Paris, France)) was used when required. The used dose was determined following veterinary recommendations depending on the dogs' glycemic curves and fructosamine levels.

2.12. Morphological and immunohistochemical analysis in dogs

Samples were fixed for 12-24 hours in 10% formalin, embedded in paraffin and sectioned. To analyze muscle integrity, cross-sections were stained with hematoxylin and eosin or rabbit anti-laminin (Z0097, Dako (Glostrup, Denmark)). Periodic acid-Schiff (PAS) staining was used to evaluate muscle glycogen content (P-5834, Sigma (St.Louis, Missouri, USA) and 109033, Merck (New Jersey, USA)). Double glucagon and insulin immunostaining were performed with mouse anti-glucagon (G2657, Sigma (St.Louis, Missouri, USA)) and guinea pig anti-insulin (I-8510, Sigma (St.Louis, Missouri, USA)) antibodies. Biotinylated horse anti-mouse (BA-2000, Vector (Burlingame, California, USA)) followed by streptavidin-conjugated Alexa488 (S-11223, Life technologies San Diego, California, USA)) and Alexa568-conjugated goat anti-guinea pig (A-11075, Life technologies San Diego, California, USA)) were used as secondary antibodies. β-cell mass was measured on four sections (2–3 □m, 200 □m apart) of pancreas biopsy or necropsy samples stained with anti-insulin and horseradish peroxydase-conjugated rabbit anti-guinea pig immunoglobulin (P0141, Dako (Glostrup, Denmark)). Pancreatic β-cell area was calculated by dividing the area of insulin-positive cells by total pancreatic area of each section.

2.13. Serum parameters determinations

2.13.1. Insulin, human C-peptide and glucagon determination

These 3 parameters were determined in serum by radioimmunoassay (RIA). A RIA is a very sensitive in vitro assay technique used to measure concentrations of antigens (for example, hormone levels in the blood) by use of antibodies. In a RIA a fixed concentration of labeled tracer antigen (125I insulin/C-peptide/Glucagon) is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited. If unlabeled antigen (the sample) is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labeled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

For the obtention of the serum in dogs the blood was taken from the foreleg cephalic vein. After taking the blood this was left coagulate 30 minutes at 4ºC and after was centrifuged at 6000g for 5 minutes and serum taken and stored at -20ºC or better -80ºC. Inclusion of Aprotinin (Sigma (St.Louis, Missouri, USA) in the tube where serum is recovered is necessary to protect samples from proteolytic destruction, c-peptide and glucagon samples, particularly. Blood samples were collected in tubes containing 250 KIU Aprotinin per ml of whole blood (25 ml of Aprotinin at a concentration of 10 KIU/ml are added in the

tube where 1 ml of whole blood will be added). This results in a final concentration of approximately 500 KIU Aprotinin per ml of serum.

Serum insulin was measured with a human insulin RIA (Millipore, Billerica, Massachusetts, USA) that has 100% cross-reactivity with canine insulin but doesn't cross react with human proinsulin (<0,2%) and rat insulin (<0,1%). The cross-reactivity with mice insulin is not stated. The sensitivity of the assay is 0.1 ng/ml.

Serum human C-peptide was determined with C-Peptide RIA (Millipore, Billerica, Massachusetts, USA) that does not cross-react with human insulin and <4% with human proinsulin. The cross-reactivity with canine and mice insulin and c-peptide is not stated. The sensitivity of the assay is 0.1 ng/ml.

Serum glucagon was measured by radioimmunoassay (Millipore, Billerica, Massachusetts, USA). The sensitivity of the assay is 0.018 ng/mL + 2 SD.

2.13.2. Blood glucose determination

Blood glucose levels were determined in mice from 5 □l of whole blood taken from the tail vein and in dogs from a drop of blood taken from the foreleg cephalic vein. The blood glucose concentration was measured using a Glucometer EliteR analyser (Bayer, Leverkusen, Germany). When fasted glycaemia is taken in dogs they were fasted during 24 hours.

2.13.3. Serum fructosamine determination

Serum fructosamine concentration was measured by nitroblue tetrazolium reduction test (Olympus, Center Valley, Pennsylvania, USA).

2.13.4. Serum tryglicerides and free fatty acids determination

Serum triglycerides and free fatty acids were determined enzymatically as previously described (Munoz et al., 2010).

2.14. Skeletal muscle glycogen determination

To determine the concentration of metabolites, skeletal muscle biopsies were clamp frozen in situ and kept at -80ºC until analysis. The concentrations of glycogen were measured in perchloric extracts, which were adjusted to pH 5 with 5M K_2CO_3 using the □-amyloglucosidase method (Bergmeyer, 1974). Afterwards, glucose was measured enzymatically (Glucose HK CP; ABX Diagnostics, Shefford, United Kingdom).

2.15. Urine analysis

Urine was analyzed by Multistix 10 SG Urinalysis Strips in a Siemens Diagnostics Clinitek Status Analyzer (Siemens Healthcare Diagnostic, Deerfield, Illinois, USA), from urine directly taken from the dogs' bladder.

2.16. Oral glucose tolerance test in dogs

Oral glucose tolerance (OGTT) tests were performed on 24-hour fasted dogs. Briefly, animals were given an oral gavage of glucose at either 3 g/kg body weight (bw) or 1.75 g/kg bw. When stated, dogs were given an oral glucose load of 1.75 g/kg bw together with a subcutaneous injection of insulin (6 IU, Lantus). Glycemia was determined at time 0, 15, 30 min and then every half hour up to 3 hours post glucose administration.

2.17. Insulin sensitivity test in mice

For intraperitoneal insulin tolerance test, awake fed mice were intraperitoneally injected with 0.75 IU/kg bw of an insulin solution (Humulin regular (Eli Lilly, Indianapolis, Indiana, USA)). Glucose concentration was determined in blood samples obtained from tail vein before and at 0, 15, 30, 45 and 60 min after the insulin injection.

2.18. Insulin release

Insulin release was performed on 24-hour fasted dogs. Dogs were fed with 30 g/kg of standard food. The samples were taken at 0, 2, 5, 10, 15, 30 and 60 minutes after dog begins to eat. At each given time blood sample was extracted independently if the dog had finished the food or not. After the blood extraction the dog was left to continue eating the remaining food. Insulin determination by RIA as explained in 2.13.1 was performed.

2.19. Exercise test

24-hour fasted dogs were subjected to 37-minute exercise under increasing speed and slope (see below) in a variable speed belt treadmill Starker Hund SH 01 Professional Treadmill (Starker Hund S.A.S., Piazzola sul Brenta, Italy). Blood glucose, heart rate and temperature were monitored before beginning the test, immediately after, and after a recovery time equal to the exercise period.

Time (min)	5	5	5	5	5	5	5	2
Incline (degrees)	0	0	2.5	5	7.5	10	0	0
Speed (km/h)	4	8	8	8	8	8	10	4

2.20. Statistical analysis

The results are expressed as the mean ± standard error of the mean (S.E.M.). The significative differences have been determined using the t-student for unpaired data considering significant *p≤0.05, very significant **p≤0.01 and extremely significant ***p≤0.001.

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