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"Effects of cyclosporine A and sirolimus on glucose and lipid metabolism – an *in vivo* rat model"

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Abstract

Cyclosporin A (CsA) and sirolimus (SRL) are immunosuppressive agents (IAs) associated with dyslipidemia, insulin resistance and new onset diabetes after transplantation (NODAT), although its molecular effects on glucose and lipid metabolism in the insulin sensitive tissues are unknown. We explored the *in vivo* IAs effects on body weight, glucose tolerance, insulin-stimulated glucose uptake on isolated adipocytes, and expression of genes involved on glucose and lipid metabolism in liver, muscle and adipose tissue from Wistar rats treated for six weeks with these agents.

Our results indicate that SRL treated animals were significantly lighter starting at week 5 (353,3 ± 5,7 vs 379,0 ± 6,4 g; $p < 0,05$) and trough week 6 (361,7 ± 5,8 vs 395,3 ± 7,0 g; $p < 0,001$), as compared to the vehicle treated group. In addition, not only did either CsA or SRL cause glucose intolerance during a GTT, and an increased lipid profile at the tissues level, but they also decreased the insulin-stimulated glucose uptake in isolated adipocytes by about 50% compared to vehicle. Furthermore, these agents caused a decrease in some of the important genes involved in insulin action, such as, IRS-1, Glut1 and Glut4, and they seem to be modulating the expression of important proteins involved in lipogenesis and gluconeogenesis.

In conclusion, these findings may suggest that cyclosporin A and sirolimus act in adipose tissue inhibiting glucose uptake, enhancing lipolysis stimulation and attenuating lipogenesis partially via down-regulation of lipogenic genes, while stimulating gluconeogenesis and lipogenesis in liver and inhibiting fatty acid oxidation in muscle, which may contribute to the development of dyslipidemia and insulin resistance associated with immunosuppressive therapy.

Keywords: Diabetes, cyclosporin A, sirolimus, lipogenesis, glucose uptake.

Resumo

Ciclosporina (CsA) e sirolimus (SRL) são agentes imunossupressores (AI) associados à dislipidemia, resistência à insulina, e ao aparecimento da *Diabetes mellitus* pós-transplante. Os seus efeitos a nível molecular são desconhecidos nos tecidos sensíveis à insulina, nomeadamente no metabolismo dos lípidos e da glicose. Neste trabalho, estudamos os efeitos dos AIs em ratos Wistar tratados *in vivo* durante 6 semanas, avaliando o peso corporal, a sua tolerância à glicose assim como a capacidade de captação da glicose após a estimulação com insulina em adipócitos isolados. Analisamos também a expressão dos genes envolvidos no metabolismo dos lípidos e da glicose em tecidos como músculo, fígado e tecido adiposo.

Os resultados obtidos indicam que os animais tratados com SRL apresentam um ganho menor de peso, sendo este resultado significativo na 5ª semana (353,3± 5,7 vs. 379,0 ±6,4 g; $p < 0,05$) e na 6ª semana (361,7 ± 5,8 vs. 395,3 ± 7,0 g; $p < 0,001$), em comparação com o grupo tratado com o solvente. Além disso, CsA ou SRL não só causaram intolerância à glicose durante o TTG, como também diminuíram em cerca de 50% a captação da glicose nos adipócitos isolados, após estimulação com insulina, e causaram um aumento do perfil lipídico ao nível dos tecidos, comparado com grupo tratado só com solvente. Por outro lado, estes agentes causaram uma diminuição em alguns dos genes importantes envolvidos na cascata da insulina, tal como, o IRS-1, Glut1 e Glut4, podendo estar a modular a expressão de proteínas envolvidas na lipogénese e gliconeogénese.

Em conclusão, estes resultados podem sugerir que a ciclosporina A e o sirolimus actuam no tecido adiposo inibindo a captação de glicose, aumentando a estimulação de lipólise e atenuando parcialmente a lipogénese através de uma regulação negativa dos genes envolvidos no metabolismo dos lípidos. Ao mesmo tempo parecem estimular a gliconeogénese e lipogénese no fígado e inibir a oxidação de ácidos gordos no músculo, podendo deste modo contribuir para o desenvolvimento de dislipidemia e resistência à insulina associada com a terapia imunossupressora.

Palavras-chave: Diabetes, cyclosporin A, sirolimus, lipogenesis, captação de glicose.

List of abbreviations

4E-BP1	Eukaryotic initiation factor 4E binding protein
ACC	Acetyl-CoA carboxylase
AMPK	AMP activated protein kinase
AS160	Rab-GTPase-activating protein
AUC	Area under the curve
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CGI	Combined glucose intolerance
ChREBP	Carbohydrate response element binding protein
CNIs	Calcineurin inhibitors
CsA	Cyclosporin A
Ctrl	Control
DGAT1	Diacylglycerol acyltrasferase 1
DM	Diabetes *mellitus*
ECF	Enhanced chemifluorescence
EDTA	Ethylenediamine tetraacid
eIF	Eukaryotic initiation factor
F-1,6-Pase	Fructose-(1,6)-biphosphatase
FAS	Fatty acid synthase
FFA	Free-fatty acids
FK506	Tacrolimus
FKBP12	FK506 binding protein

FoxO	Forkhead box O1
FPG	Fasting plasma glucose
FRB	Rapamycin-binding
G-6-Pase	Glucose-6-phosphatase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDM	Gestational diabetes
GK	Glucokinase
Glut	Glucose transporter
GS	Glycogen synthase
GSK3	Glycogen synthase kinase-3
GTT	Glucose tolerance test
HCV	Hepatitis C virus
HDL	High density lipoprotein
HLA	Human leukocyte antigens
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HMP	Hexose monophosphate
IAs	Immunosuppressive agents
IDDM	Insulin-dependent diabetes
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGF1	Insulin-like growth factor 1
IGT	Impaired Glucose Tolerance
IL	Interleukin
IP3R	Intracellular inositol 1,4,5-triphosphate receptor
IR	Insulin receptor
IRS	Insulin receptor substrate

LDL	Low density lipoprotein
LPL	Lipoprotein lipase
MHC	Histocompatibility complex
MMF	Mycophenolate mofetil
MP	Malate pyruvate
mTOR	Mammalian target of rapamicin
NFAT	Nuclear factor of activated T-cells
NIDDM	Non insulin-dependent diabetes
NODAT	New onset diabetes after transplantation
OGTT	Oral glucose tolerance test
P70 S6K	P70 ribossomal protein S6 kinase
PDK1	Phosphoinositide-dependent protein kinase 1
PEPCK	Phosphoenolpyruvate carboxykinase
PFK	Phosphofructokinase
PGC1	Proliferator-activated receptor gamma coactivator 1
PI3K	Phosphatidylinositol-3-kinase
PIP3	Phosphatidylinositol-3, 4, 5-triphosphate
PK	Pyruvate kinase
PKB	Protein kinase-B
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor gamma
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
real-time RT-PCR	Real-time reverse transcription polymerase chain reaction
S6K	Ribosomal protein S6 kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM	Standard error of the mean
SREBF1	Sterol regulatory element-binding transcription factor 1
SREBP-1	Sterol regulatory element-binding protein-1
SRL	Sirolimus
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TNFα	Tumor necrosis factor-α
TSC	Tuberous sclerosis complex
VLDL	Very low density lipoprotein
WB	Western blot
WHO	World Health Organization

Chapter I. Introduction

I - Introduction

1. Statement of the problem

In the last decades, insulin resistance and type 2 diabetes (T2D) are becoming more prevalent primarily due to worsening in life-style, such as, diet and physical activity. T2D is a multifactorial disorder of glucose metabolism whose defects include insulin resistance of liver and peripheral tissues and impaired insulin secretion. Furthermore, new onset diabetes after transplantation (NODAT) has also become a subject of interest and importance because of the increased numbers and survival rates of solid organ transplantations. Besides the fat and the muscle, the liver is also an important organ involved in the regulation of whole body glucose and lipid homeostasis. Hepatic glucose and lipid metabolic disruptions may play a central role in the onset of insulin resistance, T2D and NODAT. In addition, the development of T2D can lead to numerous secondary cardiovascular, circulatory and neuropathic complications.

2. Control of glucose and lipid metabolism by insulin

Normal plasma glucose ranges from 4 to 7 mM, this balance is kept through a tight control of glucose production by the liver, absorption by the intestine and uptake and metabolism by the peripheral tissues [1]

Insulin is the most important regulator of this metabolic balance. The increased levels of plasma glucose promote pancreatic beta-cells to secrete insulin which is one of the major hormones regulating blood glucose concentration, inducing peripheral and splanchnic glucose uptake, while, at the same time, promoting hepatic glycogen synthesis and suppressing glucose production by the liver (Figure 1) [1, 2, 3]. However, the role of insulin is not restricted to maintaining metabolic balance. Insulin signalling also stimulates cell growth, differentiation and influences life span [4]. According to Bouche and colleagues [5], the major pathways for glucose utilization consist in: i) glucose oxidation to pyruvate (glycolysis), which in turn can go through further oxidation steps in the citric acid cycle; ii) storage as glycogen (glycogen synthesis) for fast utilization at a later time and iii)

conversion to other metabolites, utilized in different pathways, like the pentose phosphate and the hexosamine biosynthesis pathway.

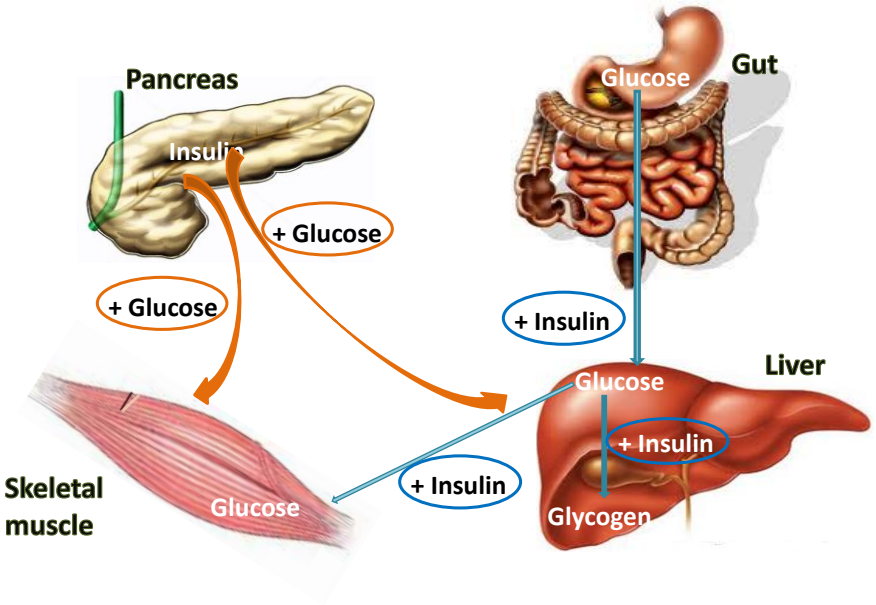

Figure 1 - Whole body glucose metabolism in postprandial conditions.

Therefore, in summary, low insulin levels (for example, in the fasting state) promotes an increase in glucose production by hepatic gluconeogenesis, glycogenolysis, and lipolysis (mobilization of free fatty acids), helped by glucagon, secreted by alpha-cells in the pancreas, while reducing glucose uptake by peripheral tissues (skeletal muscle and adipose tissue) [2]. When glucose levels rise, the balance is reversed and glucagon levels diminish, in contrast to the levels of insulin [2]. As we will discuss later, in cases of insulin resistance, this equilibrium is affected, and is characterized by higher levels of glucose and lipids in the fasting and postprandial states.

2.1. Insulin action

Insulin signalling starts with the binding of insulin to its receptor (IR). The IR is a tetrameric protein with kinase activity. It is constituted by two α- and two β-subunits (Figure 2) [1, 6, 7] The IR is part of a family of tyrosine kinase receptors that also include insulin-like growth factor 1 (IGF-1). The α-subunits inhibit β-subunits kinase activity. Insulin binding to the α-subunits causes a conformational change and auto-phosphorylation of β-subunits causing an increase of the β-subunits's kinase activity [6, 8].

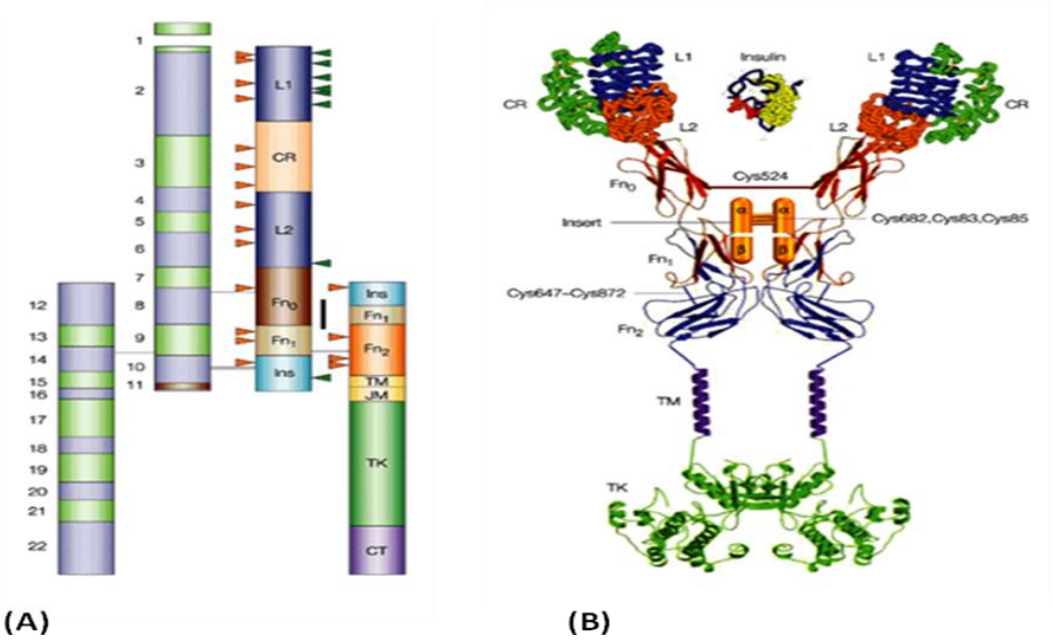

Figure 2 - Structure of α- and β-subunits of the IR. (A)On the left are represented the boundaries of the 22 exons of the IR gene and on the right the predicted boundaries of the protein modules (**B**) represents the tridimensional scheme of the IR. Extracted from De Meyts, P. et al. [6].

The signal transduction by insulin is transmitted through the tyrosine kinase activity of the insulin receptor, which catalyses the phosphorylation of cellular proteins such as members of the insulin receptor substrate (IRS) family, Shc and Cbl. IRS proteins have recently been the subject of study in explaining the action of insulin in different areas of metabolism. For instance, recent work in cellular and transgenic mouse models associates IRS-1 as a regulator of glucose metabolism, whereas IRS-2 is closer to lipid and cholesterol metabolism [9]. IRS proteins are not catalytic but rather allow interaction,

through their SH2 domains, with other downstream effectors of insulin action, such as the phosphatidylinositol-3-kinase (PI3K).

The PI3K heterodimer consists of a regulatory and catalytic subunit, p85 and p100 respectively, occurring in several isoforms. The regulatory subunit, docks phosphorylated IRS proteins and causes the catalytic subunit to release second messenger phosphatidylinositol-3, 4, 5-triphosphate (PIP3), which in turn activates further downstream signalling element phosphoinositide-dependent protein kinase 1 (PDK1) [10]. Activation of PDK1 can be downregulated by phospholipid phosphatases, like the phosphatase and tensin homolog (PTEN) protein, in turn mediating Akt/protein kinase-B (PKB) activation [11]. Akt/PKB is a serine threonine kinase, which mediates many of insulin's metabolic effects through phosphorylation of various targets including other protein kinases, transcription factors, and direct enzyme targets. [10]

Once activated, Akt/PKB mediates the stimulation of glycogen synthesis by insulin. Akt/PKB phosphorylates and inactivates glycogen synthase kinase-3 (GSK3), which is the main responsible for the inactivation of glycogen synthase (GS), leading to activation of GS, enzyme that catalyzes the conversion of UDP-glucose into glycogen, improving liver glucose uptake. GSK3 is also responsible for the inhibition of guanine nucleotide exchange factor, implicating activation of the eukaryotic initiation factor (eIF) 2B and as a consequence producing a positive effect in protein synthesis, because eIF2B controls initiation of protein translation [10, 11]. Nevertheless, the role of Akt/PKB is not limited to the glycogen synthesis or protein translation. Akt/PKB phosphorylates and activates PDE3B, a cyclic adenosine monophosphat (cAMP) phosphodiesterase isoform responsible for the decrease in cAMP concentration, resulting in decreased lipolysis. It also controls glucose transporter (Glut) 4 exocytosis by phosphorylation and inactivation of the Rab-GTPase-activating protein (AS160) and in addition it activates some protein kinase C (PKC) isoforms [10, 11, 12]. Activation of Rab small GTPases triggers the cytoskeletal reorganization necessary for translocation of Glut4, thereby increasing glucose uptake primarily in muscle and fat [13]. Akt/PKB also phosphorylates and inhibits the tuberous sclerosis complex (TSC) 2, which forms a complex with hamartin (TSC1). This complex is responsible for the inactivation of the mammalian target of rapamicin (mTOR) [14]. With the inactivation of the complex TSC2/TSC1, the mTOR pathway is effectively activated

resulting in the phosphorylation of p70 ribossomal protein S6 kinase (P70 S6K) and translation of eIF4E binding protein-1, in other words regulating protein synthesis, cell-cycle progression, lipid synthesis, and others [14]. In addition, another important role of Akt/PKB is the regulation of glucose metabolism by insulin stimulation on the forkhead box O1(FoxO) transcription factors [10]. Suppression of hepatic gluconeogenesis by insulin is modulated in vivo through the Akt/PKB-mediated phosphorylation and the relocalisation of FoxO (FoxO1, FoxO2 and FoxO3) from the nucleus to the cytoplasm, inhibiting its function in gluconeogenesis [4, 10, 11, 12, 15]. The insulin signalling pathway is shown in details on Figure 3.

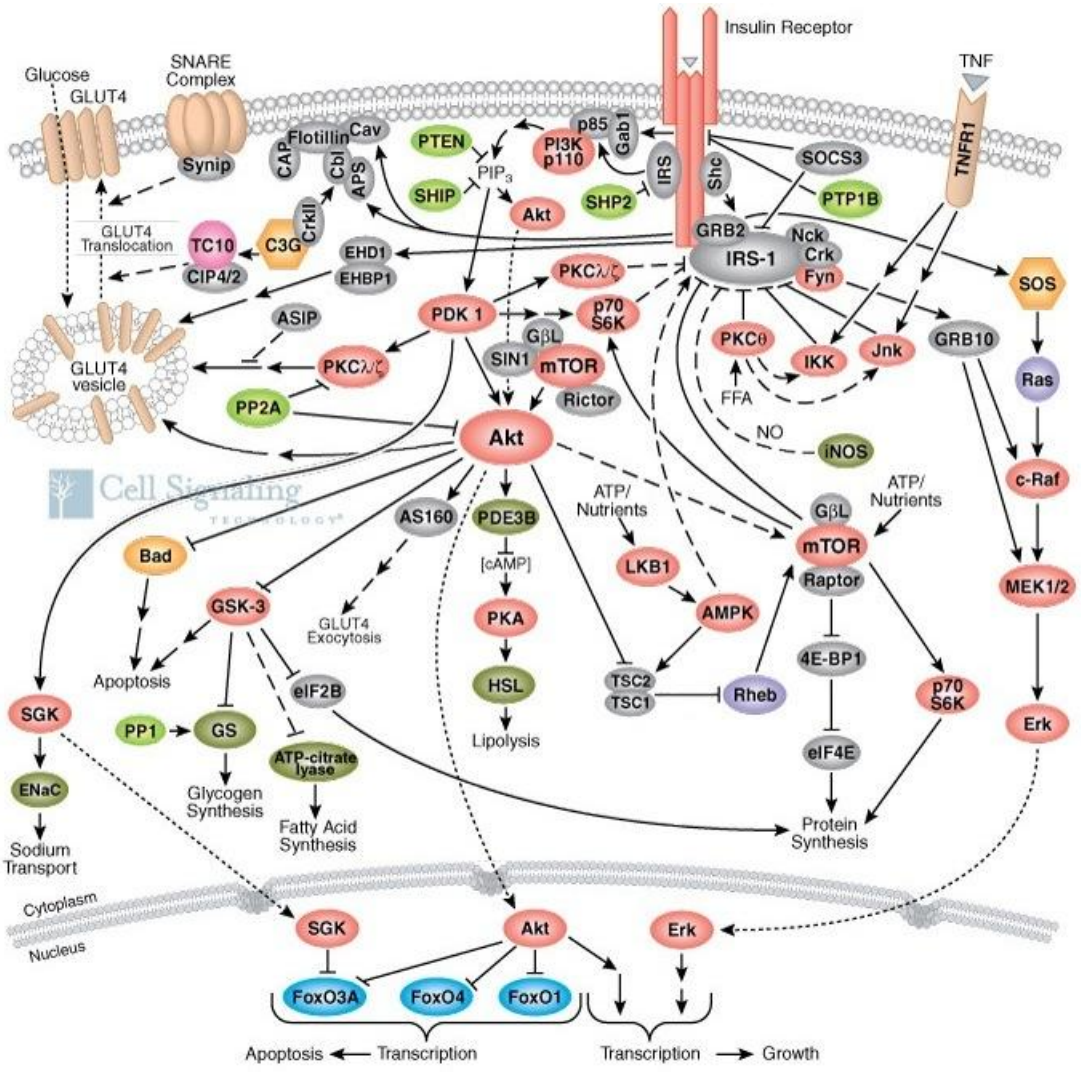

Figure 3 - Insulin signalling pathway. Extracted from ref. [16].

Furthermore, Akt/PKB is suggested to be involved in almost all of the actions of insulin as summarized on Figure 4. Further knowledge in Akt/PKB action may render possible new approaches for controlling diabetes without the usage of insulin.

Figure 4 - Summary of the Akt/PKB action in insulin-responsive tissues.

Moreover, current research explains the divergent regulation of gluconeogenesis and fatty acid metabolism in the liver in response to insulin as branches of the insulin signalling pathway at the level of the IRS molecules; partial IRS-1 downregulation by short hairpin RNA results in upregulation of the aforementioned gluconeogenic enzymes, while IRS-2 downregulation causes upregulation of lipogenic enzymes, as the sterol regulatory element-binding protein 1 c (SREBP-1c) and fatty acid synthase (FAS) [17]. The divergent functions of these signalling molecules are unified in the action of transcription factor FoxO1 (Figure 5), which induces hepatic lipid accumulation by increasing triglyceride synthesis while also inducing insulin-independent Akt/PKB expression which in turn causes decreased glucose production [17, 18]. Fox genes encode a subgroup of helix–turn–helix class of remarkably conserved transcription factors (>50 genes in man), controlling numerous biological processes including development,

organogenesis, cell differentiation, cell cycle control, apoptosis, and functions as diverse as speech and language development and gluconeogenesis [19]. In the absence of insulin, FoxO1 positively controls the expression of genes involved in gluconeogenesis enhancing transcription of genes involved in gluconeogenesis, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P) [18]. Upon binding to its receptor, insulin triggers the phosphorylation of FoxO1 via phosphatidylinositol 3-kinase and Akt/PKB [20]. Phosphorylated FoxO1 is no longer retained in the nucleus and is unable to bind peroxisome proliferator-activated receptor-γ coactivator 1 (PGC1) α and thus becomes transcriptionally inactive. This is the basis of the negative control of gluconeogenesis by insulin. In contrast, insulin seems to have no effect on PGC1α expression [18].

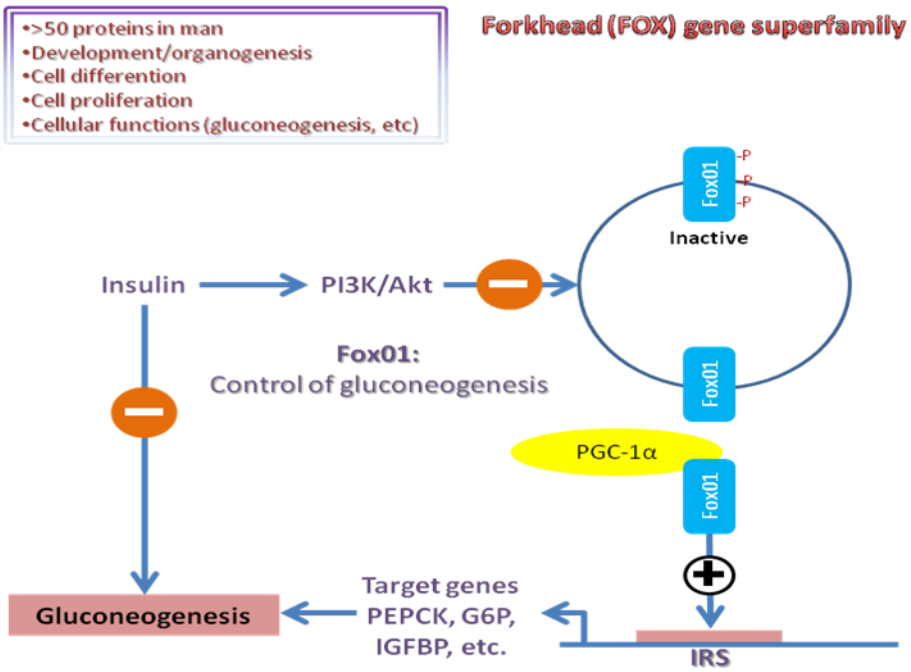

Figure 5 - The actions of FoxO1. Abbreviations: phosphatidylinositol-3-kinase (PI3K); peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1 α); insulin receptor substrate (IRS); phosphoenolpyruvate carboxykinase (PEPCK); glucose-6-phosphatase (G6P); Insulin-like Growth Factor Binding Proteins (IGFBP).

In addition, insulin regulates the expression of some important and rate-limiting enzymes, involved in glycolysis, as well as, in gluconeogenesis, promoting glucose utilization and storage in the form of glycogen and lipids [5]. Insulin activates glycogen synthase and citrate lyase by influencing their phosphorylation state. On the other hand it inhibits the expression of some gluconeogenic enzymes and transcription factors, such as fructose-(1,6)-biphosphatase (F-1,6-Pase), PEPCK and glucose-6-phosphatase (G-6-Pase), while activating glycolitic and lipogenic enzymes, such as glucokinase (GK), pyruvate kinase (PK), FAS and acetyl-CoA carboxylase (ACC) as well as the transcription factor SREBP, as shown on Figure 6 [1, 5].

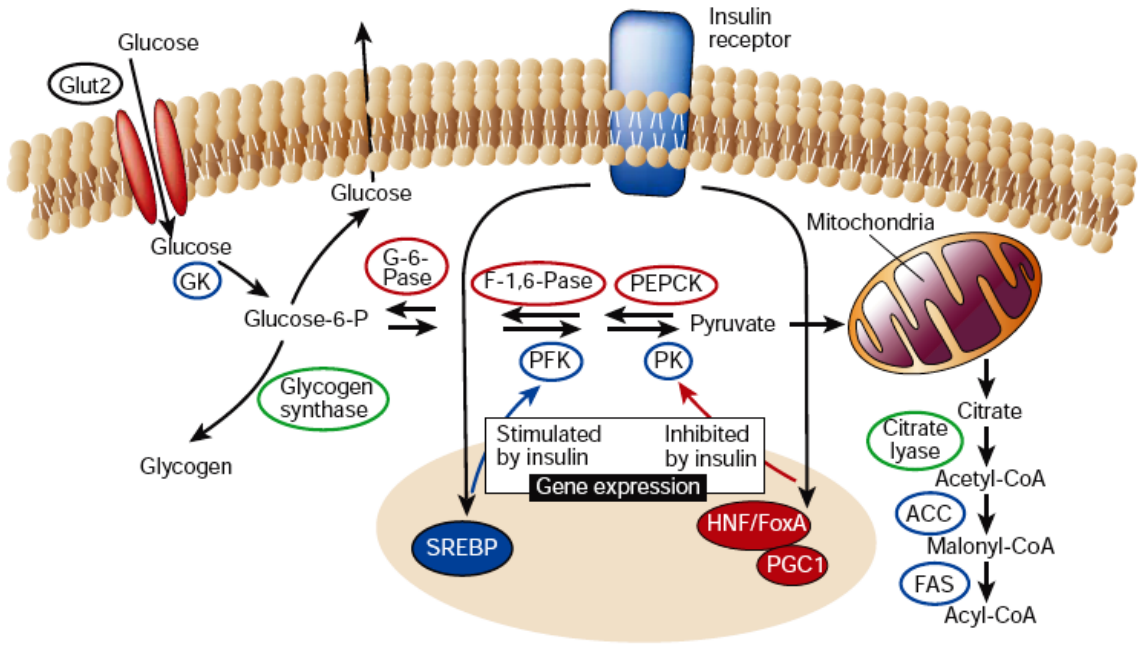

Figure 6 - Hepatic glucose utilization. In red: gluconeogenic enzymes and transcription factors; in blue: glycolitic and lipogenic enzymes. Abbreviations: Sterol regulatory element-binding proteins (SREBP); Glucokinase (GK); glucose-6-phosphatase (G-6-Pase); fructose-1,6- biphosphatase (F-1,6-Pase); phosphoenolpyruvate carboxylase (PEPCK); phosphofructokinase (PFK); pyruvate kinase (PK); acetyl-CoA carboxylase (ACC); fatty-acid synthase (FAS). Adapted from Cade, W.T. et al. [21].

2.2. Gluconeogenesis

Gluconeogenesis is the biosynthesis of new glucose from molecules that are not carbohydrates (Figure 7), (i.e. no glucose from glycogen). It operates as the biosynthetic pathway responsible for countering glycolytic breakdown of glucose, and has often been described as the "reverse of glycolysis" [5]. One important function of gluconeogenesis is the ability to convert some metabolic products, like lactate or glycerol into glucose, when glucose dietary sources are not available. This is very useful in the fasted state to control glycemia. The liver is the major site of gluconeogenesis, however, the kidney also has an important part to play in this pathway [5].

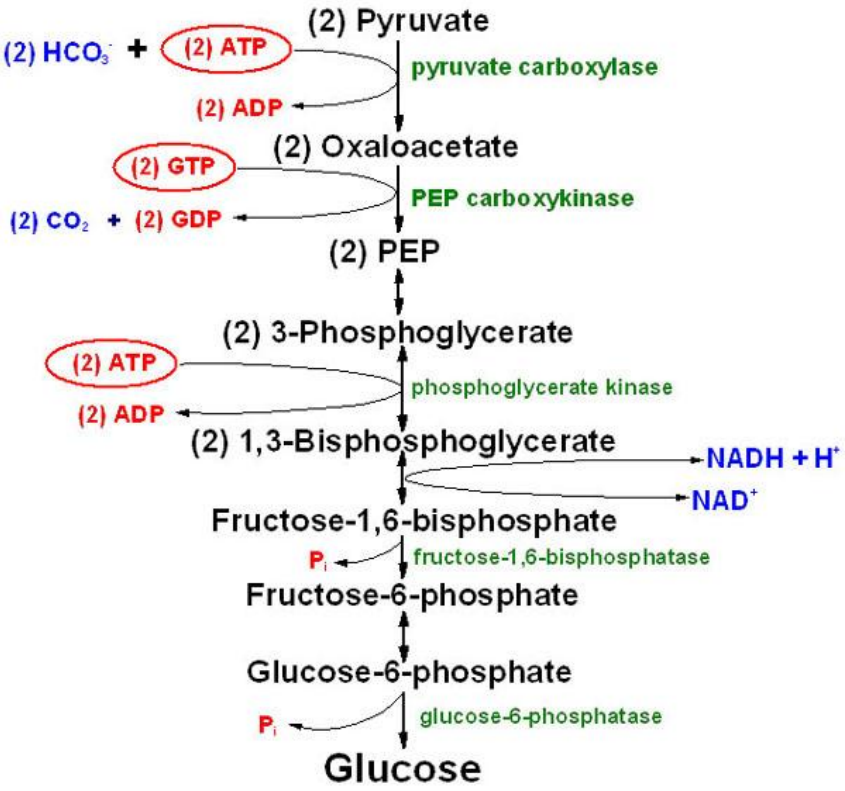

Figure 7 - Relevant reactions of gluconeogenesis. The enzymes of the 3 bypass steps are indicated in green along with phosphoglycerate kinase. In hepatocytes the glucose-6-phosphatase reactions allows the liver to supply the blood with free glucose. Because of the high K_m of the liver glucokinase, most of the glucose will not be phosphorylated and will flow down its concentration gradient out of hepatocytes and into the blood. Adapted from King, M.W. [22].

The production of glucose from other metabolites is necessary particularly to be used as a fuel source by the brain, testes, erythrocytes and kidney medulla since glucose is the sole energy source for these organs [23]. During starvation, however, the brain can derive energy from ketone bodies that are converted to acetyl-CoA [24]. The primary carbon sources used for gluconeogenesis are pyruvate, lactate, glycerol, and the amino acids alanine and glutamine [5].

Finally, PGC1α seems to have an important role in the regulation of gluconeogenesis. PGC1α is also responsible for mitochondrial biosynthesis, being involved in β-oxidation and in the expression of gluconeogenic genes [1, 5, 11, 15, 25]. Glucose metabolism is at the heart of Type 2 Diabetes, with the characteristic hyperglycemia resulting from decreased glucose uptake into insulin-sensitive tissues, primarily in muscle and fat [5]. As discussed below, in the pathophysiologic state of T2D, as well as during immunosuppressant treatments, the equilibrium between gluconeogenesis and glycogenesis is disturbed.

2.3. Lipogenesis

Fat accumulation is determined by the balance between fat synthesis (lipogenesis) and fat breakdown (lipolysis, fatty acid oxidation). Lipogenesis is the process by which acetyl-CoA is converted to fats and it takes place in both liver and adipose tissue. The former is an intermediate in metabolism of simple sugars, such as glucose. Through lipogenesis, energy can be efficiently stored in the form of fats. Lipogenesis encompasses the processes of fatty acid synthesis and subsequent triglyceride synthesis (when fatty acids are esterified with glycerol to form fats). Plasma glucose levels stimulate lipogenesis via several mechanisms [26]. First, glucose itself is a substrate for lipogenesis: as it's glycolytically converted to acetyl-CoA, glucose promotes fatty acid synthesis [26]. Secondly, glucose induces the expression of lipogenic genes, the mechanisms of which are explained below. Finally, glucose increases lipogenesis by stimulating the release of insulin and inhibiting the release of glucagon from the alpha-cells of pancreas [26]. The effects of nutrients and hormones on the expression of lipogenic genes are mostly mediated by SREBP-1, and in adipose tissues by peroxisome proliferator-activated receptor gamma

(PPARγ) [26]. These transcription factors are responsible for the expression of genes encoding some important enzymes of lipogenesis: pyruvate dehydrogenase, fatty acid synthase and acetyl CoA carboxylase [1, 27]. Thus, insulin signalling induces SREBP expression (by PI3K/Akt/PKB pathway), promoting the synthesis of lipids (Figure 8).

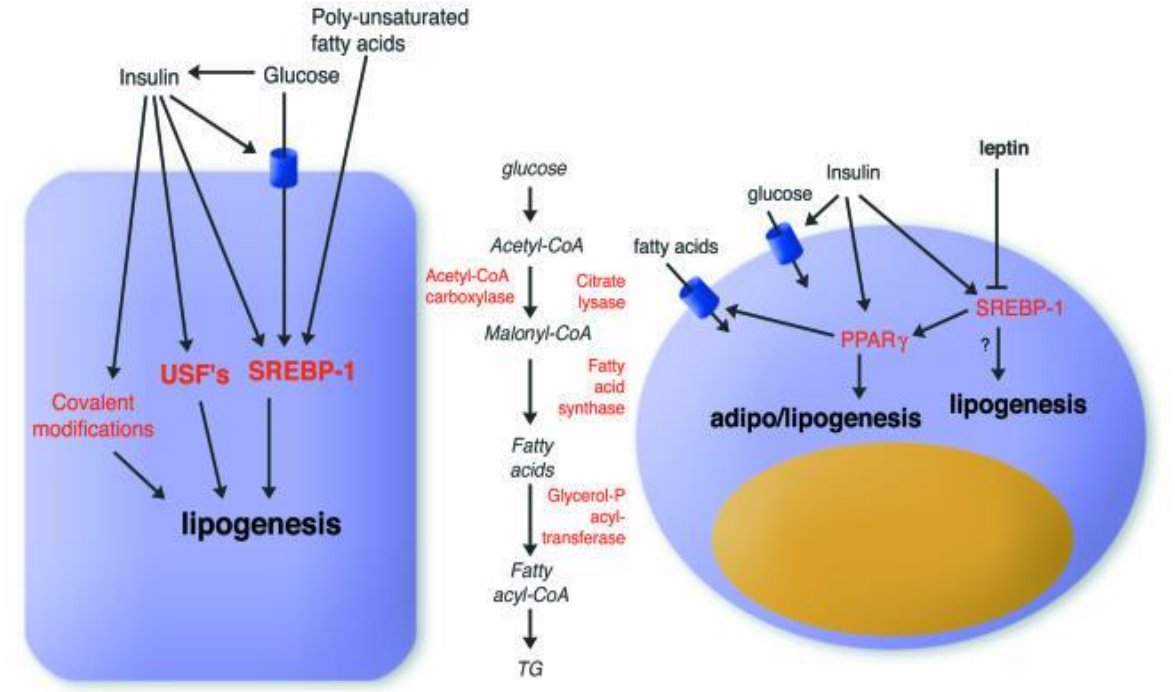

Figure 8 - Mechanisms of nutritional and hormonal regulation of lipogenesis in hepatocytes (left) and adipocytes (right). The effects of nutrients and hormones on the expression of lipogenic genes are mostly mediated in liver by SREBP-1 and in adipose tissue, by PPARγ. Lipogenesis entails a number of discrete steps, shown in the middle, which are controlled via allosteric interactions, by covalent modification and via changes in gene expression. Extracted from Kersten, S. [26].

However, there are reports of over-expression of SREBP in cases of insulin resistance in liver (where insulin signalling is reduced) [27, 28, 29] and an effect of endoplasmatic reticulum stress in the activation of SREBP as suggested by Ferré and Foufelle [27]. In the fed state glucose and insulin coordinate hepatic lipogenesis and glycolysis by regulating SREBP, as well as carbohydrate response element binding protein (ChREBP). ChREBP share with SREBP the same lipogenic genes, genes related with the hexose monophosphate (HMP) and the malate pyruvate (MP) shunts. Both insulin and glucose are potent factors in inducing the transcription of these key enzymes (Figure 9) [30].

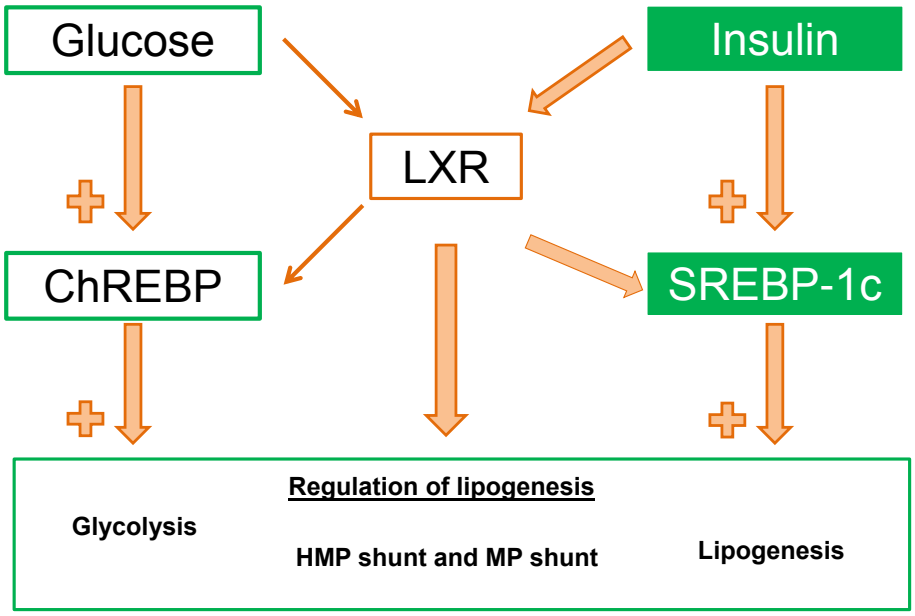

Figure 9 - Glucose and insulin activate transcription factors and regulate *de novo* lipogenesis in liver. Glucose activates ChREBP and insulin activates SREBP-1c and LXR. Abbreviations: LXR, liver protein X receptor; SREBP, sterol regulatory element binding protein 1 c; ChREBP, carbohydrate regulatory element binding protein; HMP, hexose monophosphate; MP, malate pyruvate shunt.

In addition, the high fat content of the typical western diet is considered to be an important factor in the development of T2D [31]. In this setting, there is an initial phase often referred to as the "prediabetic state" which is characterized by weight gain, an increase in body fat and the development of glucose intolerance. In addition to an overall gain in body fat, there is increased deposition of ectopic triglycerides (TGs), particularly in liver and skeletal muscle [32]. Inhibition of TGs biosynthetic enzymes has been suggested to be one of the potential strategies to treat these disorders [33]. For istance, the enzyme catalyzing the final and committed step in the TGs biosynthetic pathway is the diacylglycerol acyltransferase 1 (DGAT1), one of several enzymes with DGAT activity that were cloned and characterized at the molecular level [33].

Since these lipid pools may play a key role in the development of insulin resistance at an early and possibly reversible stage of T2D, there is an increased interest in the study of ectopic lipid dynamics both in order to better understand the pathogenesis of insulin resistance and also as a clinical marker for identifying people that may be at an elevated risk of developing T2D, such as first degree relatives of type 2 diabetic patients.

The development of insulin resistance and diabetes is closely linked with the disruption of hepatic glucose and lipid metabolism. Classical approaches of hepatic metabolism assessment include rather invasive techniques such as liver biopsies or hepatic venous catheterization protocols. However, the development of techniques involving stable isotopes and Nuclear Magnetic Resonance combined with the application of safe and noninvasive methods, such as Magnetic Resonance Imaging and Spectroscopy have made it possible to more easily assess hepatic glucose and lipid metabolism *in vivo* in both animals and humans.

3. Diabetes

3.1. Definition and incidence of diabetes

The term Diabetes *Mellitus* (DM) describes a metabolic disorder of multiple etiology, characterized by chronic hyperglycemia, with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both [34]. The etiology of diabetes *mellitus* comprises a complex interaction of genetics, environmental factors, such as, life-style choices. Its pathogenesis includes reduced insulin secretion, decreased glucose utilization and increased glucose production [35]. The major classes of DM, as explained in detail below, are T2D and type 1 diabetes (T1D).

Diabetes *mellitus* is a widespread and growing public health problem reaching epidemic proportions in many parts of the world. Diabetes has reached a 6,4% worldwide incidence, affecting 285 million adults, in 2010, and it is estimated to increase to 7,7%, 439 million adults, by 2030 [36]. Nowadays 346 million people worldwide have diabetes [37] and the growing prevalence is highly associated with the increasingly sedentary life-style, together with access to energy-rich diets in genetically susceptible individuals. Moreover, the secondary complications of diabetes, including cardiovascular and microvascular dysfunction, place a large burden on health care (i.e., heart failure, stroke, blindness and end-stage renal disease) [21]. Overall, treatment of diabetes and its secondary complications are estimated to consume up to 15% of the world's healthcare budget [38]. Additionally, the economic burden of diabetes in the United States alone, in 2002, was

estimated to be $132 billion, hence its economic impact is considerable [39]. Diabetes UK reports that one in 10 people admitted to hospital have diabetes. Approximately 15% of deaths per year are caused by the disease [40]. Diabetes is directly responsible for considerable morbidity and mortality, accounting for 5,2% of worldwide mortality in 2000 [41]. The International Diabetes Federation (IDF) estimated that in 2011 the five countries with the largest numbers of people with diabetes were China, India, the United States of America, Russia and Brazil [42].

3.2. Risk factors and warning signs of diabetes

The risk factors for T1D are still being investigated. However, having a family member with T1D increases the risks for developing the condition, as do the presence of some genetic factors, such as variation in Human Leukocyte Antigens (HLA) genes [43]. Environmental factors, increased height and weight development, increased maternal age at delivery, and exposure to some viral infections have also been linked to the risk of developing the disease. In addition, obesity, diet and physical inactivity, increasing age, insulin resistance, family history and ethnicity have also been associated with type 2 diabetes [44]. Moreover, as discussed below, pregnant women who are overweight, have been diagnosed with impaired glucose tolerance (IGT), or have a family history of diabetes, are at increased risk of developing gestational diabetes *mellitus* (GDM). One among other warning signs for diabetes is frequent urination and the consequent excessive thirst. Indeed, the name “diabetes” has a Greece origin and means “to siphon”, reflecting how diabetes seemed to rapidly drain fluid from the affected individual. [45, 46]. Individuals can experience different warning signs for diabetes, and sometimes there may be no obvious warning, but some of the most common signs experienced will be summarized in Figure 10. The onset of T1D is usually sudden and dramatic while the symptoms can often be mild or absent in people with T2D, making this type of diabetes gradual in onset and harder to detect.

Figure 10 - Major signs of diabetes.

3.3. Classification of Diabetes

In contrast with the past [47], where diabetes *mellitus* was classified based on the onset age or therapy type, nowadays DM is classified on the basis of the pathogenic process that results on hyperglycemia [2]. In 1980, two major classes of DM were proposed by the World Health Organization (WHO) Expert Committee on Diabetes *Mellitus*. These were named IDDM ("insulin-dependent diabetes") or Type 1, and NIDDM (non insulin-dependent diabetes) or Type 2. This was then modified in 1985, introducing Malnutrition-related Diabetes *Mellitus*. In both the 1980 and the 1985 reports other classes of diabetes were also included, such as, Impaired Glucose Tolerance (IGT), as well as, GD [47]. More recently, according to the American Diabetes Association [48], diabetes can be classified into 4 main categories; type 1 diabetes, type 2 diabetes, gestational diabetes and other specific types (see American Diabetes 2011). From these, the more frequent are T1D and T2D (Table 1).

Table 1 - Etiologic classification of diabetes *mellitus* [34]

I. Type 1 diabetes (T1D)
Pancreatic beta-cell destruction, usually leading to absolute insulin deficiency
A. Immune mediated
B. Idiopathic
II. Type 2 diabetes (T2D)
Predominant insulin resistance with relative insulin deficiency
III. Oher specific types
A. Genetic defects of beta-cell function
B. Genetic defects of insulin action
C. Diseases of the exocrine pancreas
D. Endrocrinopathies
E. Drug or chemical-induced Immunosuppression and NODAT
F. Infections
G. Uncommon forms of immune-mediated diabetes
H. Other genetic syndromes sometimes associated with diabetes
IV. Gestational diabetes *mellitus* (GDM)

3.3.1. Impaired glucose regulation, impaired glucose tolerance and impaired fasting glucose

The Expert Committee on the Diagnosis and Classification of Diabetes *Mellitus* extended the concept in 1997 by recognizing patients with impaired fasting glucose (IFG) in addition to those with IGT [49]. Both categories were referred to as pre-diabetes and are considered substantial risk factors for progression to diabetes. Moreover, microvascular

complications, including retinopathy, chronic kidney disease, neuropathy, and cardiovascular disease have been associated with pre-diabetes [50, 51].

The Expert Committee on the Diagnosis and Classification of Diabetes *Mellitus* in 1997 and the WHO in 1998 recommended that the fasting plasma glucose (FPG) threshold be reduced from 140 mg/dL (7.8 mmol/L) to 126 mg/dL (7.0 mmol/L) for diagnosing diabetes [49, 52]. In addition, the Expert Committee and the WHO defined the 2 intermediate states of abnormal glucose regulation between normal glucose homeostasis and diabetes. While IGT is confirmed by a 2-hour plasma glucose level between 140 and 199 mg/dL (7.8–11.1 mmol/L) after a 75-g oral glucose tolerance test (OGTT), the IFG is confirmed by a FPG level between 110 and 125 mg/dL (6.1–6.9 mmol/L). It should be stated, however, that IFG and IGT are not interchangeable and represent different abnormalities of glucose regulation, one in the fasting state and one in the post-prandial state [53]. Moreover, individuals with pre-diabetes have approximately a 30% chance of developing type 2 diabetes over a 10-year period [54, 55]. Skeletal muscle insulin resistance, with concomitant beta-cell dysfunction, characterizes individuals with IGT, whereas hepatic insulin resistance and first phase insulin secretion deficiency describes those with IFG [56]. Combinations of skeletal muscle and hepatic insulin resistance, as well as, beta-cell dysfunction depict individuals with combined glucose intolerance [56]. In addition, diagnosed, undiagnosed diabetes, and impaired fasting glucose states are associated with increased age, body mass index (BMI), waist-to-hip ratio, systolic blood pressure, total cholesterol, and serum creatinine levels [57].

3.3.2. Type 1 Diabetes

Type 1 diabetes is sometimes called insulin-dependent, immune-mediated or juvenile-onset diabetes. It develops when the immune system in the body attacks insulin-producing beta-cells in the pancreas and destroys them [58]. The reason why this occurs is not fully understood. People with T1D produce very little or no insulin. Therefore, when beta-cells no longer make insulin, blood glucose cannot enter the insulin responsive cells, such as muscle and fat, to be used for energy, leaving high levels of glucose in circulation, causing a state of hyperglycemia.

3.3.2.1. Epidemiology

The disease can affect people of any age, but usually occurs in children or young adults. Until recently, T1D was the most common form of diabetes diagnosed before the age of 30; however, it can also develop in adults (latent autoimmune diabetes of adulthood, which often initially appears to be T2D) [59]. People with this form of diabetes need injections of insulin every day in order to control the levels of glucose in the blood. Type 1 diabetes accounts for <10% of all cases of DM and the prevalence varies between countries and between regions in the same country [60]. In 2000, Karvonen et al., investigated and monitored the patterns in the incidence of childhood T1D worldwide [61]. A very high incidence (>20/100,000 per year) was found in Sardinia, Finland, Sweden, Norway, Portugal, the U.K., Canada, and New Zealand [61, 62]. The lowest incidence (<1/100,000 per year) was found in the populations from China and South America [61]. In Portugal the prevalence of T1D (0-19 years old), in 2009, was 0.12%, the evolution from 2000 to 2009 was on average 11.2 new cases per 100 000 individuals [63]. In most populations, the incidence increased with age and was the highest among children 10–14 years of age [64]. The variation seems to follow ethnic and racial distribution in the world population. The explanation for these wide risk disparities within ethnic groups may lie in differences in genetic admixture or environmental/behavioral factors [61].

3.3.2.2. Pathophysiology

One of the most intriguing aspects of T1D is the selective attack on beta-cells even though they have the same embryological origin and share most of the proteins with other cell types in the islet [58]. There are probably two main causes for T1D, one is genetic background and the other environmental effects. There are some genetic variations that characterize T1D patients, for example, the HLA locus, containing genes expressing some histocompatibility complex (MHC) molecules [43, 65]. The environmental causes of T1D include viral infections, dietary factors, vaccination and toxins that can have influence in the disease development [65]. Nevertheless, enteroviruses seem to have an important role in the pathogenesis of T1D [66].

3.3.3. Type 2 Diabetes

Type 2 diabetes is sometimes called non-insulin dependent diabetes or adult-onset diabetes, and it is one of the most frequent forms of diabetes, occurring in about 90% of all cases of diabetes [67]. When the body does not produce enough insulin or it does not use it properly or efficiently, T2D develops. It is characterized by insulin resistance and relative insulin deficiency either of which may be present at the time that diabetes becomes clinically manifest.

T2D is characterized by different disorders, normally sharing insulin resistance, impaired insulin secretion and increased glucose production. [58] The combination of hypertension, dyslipidemia, insulin resistance, hyperinsulinemia, glucose intolerance, and obesity, particularly central obesity have been termed the "metabolic syndrome." It has been proposed that this syndrome is a powerful determinant of diabetes and cardiovascular disease [68].

3.3.3.1. Epidemiology

The diagnosis of T2D usually occurs after the age of 40 but can occur earlier, especially in populations with high diabetes prevalence [67]. The proportion of cases of T2D in people with impaired glucose tolerance or impaired fasting glucose levels was reduced in landmark trials from China, Finland, and the United States by up to 33%, 50%, and 58%, respectively, through lifestyle changes (increased exercise, weight loss) or pharmacotherapy, or both, although changes may be more modest in a non-trial population [69]. However, Canada currently estimates that 6% of male and 5% of female Canadians older than 12 years of age have been diagnosed with diabetes [70] and unfortunately the incidence is expected to increase further in all age groups over the next decades, possibly affecting 2.5 million Canadians by the year 2016 [71]. In Portugal, the prevalence of T2D in 2009 was 11.7 %, corresponding to 905 thousand patients [63].

3.3.3.2. Insulin resistance

Insulin resistance is characterized by a diminished capacity to deactivate the liver glucose production in the fasting state and/or impairment in glucose utilization by insulin-sensitive tissues (i.e. muscle, liver and fat). The pancreas compensates insulin resistance by rising insulin production, which eventually will normalize glucose levels [58]. Insulin action is also impaired in insulin resistance states; including in young healthy relatives of T2D patients, there is evidence of decreased insulin-stimulation of IRS-1 tyrosine phosphorylation and IR activity. Insulin resistance with low cellular IRS-1 expression is also associated with low Glut4 expression and impaired insulin-stimulated glucose transport. [72] The main proposed mechanisms for insulin resistance are the attenuation of the insulin signalling through serine phosphorylation of IRS-1, and second with degradation of IRS-1 [73, 74]

Furthermore, other possible mechanisms for insulin resistance might be mitochondrial dysfunction [75]. T2D, associated with obesity and mitochondria, have an important role in fuel utilization and energy production, consequently one would expect a correlation between a defective mitochondrial function and the pathophysiology of T2D [75]. In fact there is a strong correlation between impaired mitochondrial function and T2D, and this concept would explain the excess triglyceride accumulation and the impairment of insulin-mediated glucose uptake because of the reduced β-oxidation and ATP production, however this relationship needs further investigation [76, 77]. Until recently, the importance of adipose tissue in the regulation of glucose metabolism was neglected. Increased adipocyte mass, associated with obesity, is responsible for increased free-fatty acids (FFA) circulating levels that can impair skeletal muscle glucose utilization, promote liver glucose production and as mentioned before, impair beta-cell function [78]. Adipocytes produce a very broad number of biologic products important to the pathogenic processes of T2D, such as, adiponectin [79], retinol binding protein 4 [80], resistin [81] and tumor necrosis factor α (TNFα), among others [82]. These adipokines can regulate body weight, appetite, energy expenditure and insulin sensitivity. Finally, the capacity of insulin to suppress liver glucose production fails (gluconeogenesis), resulting in fast hyperinsulinemia that is accompanied by a decrease in glycogen synthesis by the liver in the postprandial state [5]. Caused by adipose tissue insulin resistance, the flux of FFA from

the adipocytes to the liver is high, raising the very low density lipoprotein (VLDL) and triglyceride synthesis, which leads to the characteristic dyslipidemia of T2D [31].

3.3.4. Gestational Diabetes

During pregnancy, appropriate nutrient flow to the fetus is ensured by moderate peripheral insulin resistance and hyperinsulinemia. The pancreas adapts by increasing insulin biosynthesis, enhancing glucose-stimulated insulin secretion and increasing beta-cell mass. When beta-cells fail to adapt, glucose levels rise to pathological levels, leading to gestational diabetes [83] that is any degree of glucose intolerance with onset or first recognition during pregnancy [84,85]. The prevalence of GDM in the developed world has increased at an alarming rate over the last few decades.

3.3.4.1. Epidemiology

GDM develops in one of 25 pregnancies worldwide and is associated with complications in the period immediately before and after birth [67]. The incidence of T2D in women with previously diagnosed GDM (pGDM) who were examined six weeks to 28 years postpartum was estimated to range from 2.6 to 70% [86, 87]. GDM usually disappears after pregnancy but researchers found that women with pGDM have an 18–50% higher risk of developing type 2 diabetes *mellitus* within 5 years following pregnancy [88, 89]. A study by Stone et al. reports that in Victoria the estimate incidence of gestational diabetes was 3.6% in 1996 [90]. Women with gestational diabetes had increased rates of hypertension, pre-eclampsia, induced labour, and interventional delivery [90]. Their offspring had a higher risk of macrosomia, neonatal jaundice and hyaline membrane disease [90]. In the US, GDM occurs more often among African American, Hispanic/Latino Americans and American Indians comparing with White Americans [91]. In 2009, in Portugal, it was observed that 3,9% of all pregnant women delivering in the public hospitals, in a total of 80% of all delivers had GDM [63].

3.3.4.2. Pathophysiology

Pregnancy is characterized by progressive insulin resistance, starting near mid-pregnancy reaching levels close to the insulin resistance present in T2D patients in the third trimester. The increased insulin resistance during pregnancy has been attributed to a combination of adipose tissue increase, adipokines effects, cortisol and gestational hormones, but more recent data have shown that cytokines may also be involved in this process [91]. Nevertheless, in normal pregnancy, glucose levels are maintained by the increase in insulin secretion. The most significant maternal risk is the development of the metabolic syndrome characterized by central obesity, dyslipidemia, and insulin resistance, which predisposes to increased risk for coronary artery disease, stroke, and T2D later in life [92]. GDM shares with T2D most of the common characteristics of the pathology, such as pancreatic beta-cell failure and chronic insulin resistance [96]. Like T2D, GDM is a multifactorial disease, involving insulin resistance, mechanisms that diminish insulin-signalling trough the IR. To note that, by correlating insulin resistance with beta-cell responses, several studies performed in women before or after pregnancy revealed the presence of beta-cell dysfunction [93-95].

Furthermore, in women with GDM the expression of IRS-1 is reduced while p85α levels are increased, affecting insulin signalling downstream, in both skeletal muscle and adipose tissue [95]. Increased IRS-1 serine phosphorylation is an additional factor reducing insulin signalling in GDM patients [95]. Pro-inflammatory regulators, such as, TNFα that is secreted by adipose tissue is responsible for various stress responses. TNF-α causes the increase in serine phosphorylation of IRS-1 and reduces IR tyrosine kinase activity, causing the reduction of Glut4 translocation, resulting in lower glucose uptake capacity by cells [95, 96]. TNFα also mediates suppression of adiponectin transcription [95, 96]. Adiponectin is a secreted globular protein synthesized exclusively by adipocytes and it is implicated in the pathogenesis of insulin resistance and insulin sensitization presumably stimulating glucose uptake in skeletal muscle and reducing hepatic glucose production through its effect on AMP activated protein kinase (AMPK) [95, 97]. Indeed, plasma adiponectin concentration has been found lower in obesity, T2D, and GDM [98, 99]. Resistin also has an important role in the GDM etiology. It's expressed by monocytes, macrophages and by the placenta during pregnancy, although the physiologic influence of

resistin in the pathogenesis of human insulin resistance seems to be lower compared with rodents. There is, however, evidence for its involvement in the origin of insulin resistance characteristic of GDM [100]. The molecular changes in adipose tissue during pregnancy include a reduction in the transcription factor PPAR-γ, indicating a metabolic switch to lipolysis, increasing postprandial FFA and hepatic glucose production, resulting in a greater fuel availability to the fetus and acceleration of the insulin resistant state [101].

Therefore, the pathophysiology of GDM comprises a combination of pro-inflammatory mediators acting together with placental hormones, reducing adiponectin secretion and increasing lipolysis, originating severe liver, muscle, and adipose tissue insulin resistance. Moreover, other specific types of diabetes result from specific genetic conditions (such as maturity-onset diabetes of youth), surgery, medications, infections, pancreatic disease, and other illnesses, and account for 1 to 5% of all diagnosed cases [102].

3.3.5. New onset diabetes after transplantation

The calcineurin inhibitors (CNIs), cyclosporin A (CsA) and tacrolimus (FK506), as well as the mTOR inhibitors, everolimus and sirolimus (SRL), are immunosuppressive agents (IAs) frequently used to prevent rejection after solid organ transplantation and treatment of autoimmune diseases [103]. However, standard recommended doses are associated with the development of hypertension, hyperlipidemia, insulin resistance and diabetes [104, 105]. By definition, NODAT refers to abnormal glucose metabolism that is detected after transplantation, although it should be clearly pointed out that the disease (and hence its management) begins with numerous factors that antedate the transplant. In addition, a number of patients who develop NODAT have shown evidence of glucose intolerance or insulin resistance before transplantation [106]. The development of NODAT is largely driven by an imbalance between insulin production and insulin required by target tissues to regulate effectively fasting glucose production and postprandial glucose disposal. It follows that transplant-specific exposure to immunosuppression contributes to the development of NODAT via these two mechanisms [106]. Experimental and clinical studies suggest that IAs increase serum levels of cholesterol, triglycerides, VLDL and FFA

in a dose dependent-manner, and approximately 60% of patients treated, are reported to have abnormal lipid profiles [107]. Although immunosuppressive therapy has been strongly associated with dyslipidemia and NODAT, the underlying mechanisms related to impaired glucose and lipid metabolism have not been fully elucidated. NODAT is therefore, a serious complication of organ transplantation and is associated with an increased risk of cardiovascular morbidity and mortality [106, 108, 109]. In addition, its adverse effects on organ rejection and quality of life of the patient are also a recent and rapidly growing concern. Several NODAT risk factors have been identified, such as age, ethnicity, obesity, metabolic syndrome, hepatitis C infection, and immunosuppressive regimens[108-112]. Other risk factors are summarized on the Figure 11.

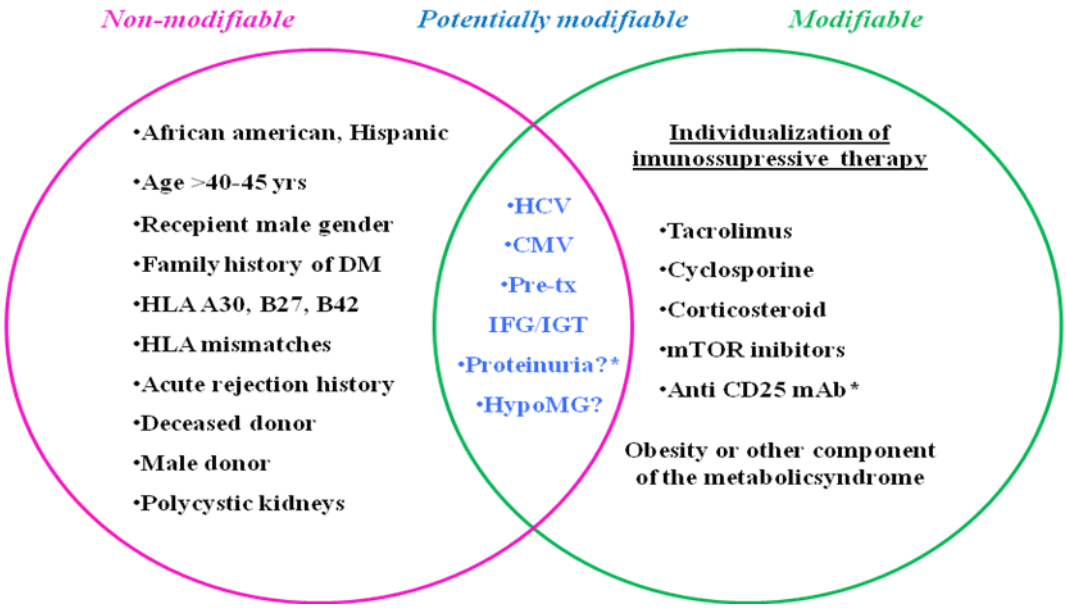

Figure 11 - Risk factors for NODAT. Abbreviations: Anti CD25 mAb, Anti CD25 monoclonal antibody; CMV, cytomegalovirus; HCV, hepatitis C; HypoMg, hypomagnesemia; Pre-Tx, pre-transplant. Adapted from ref.[113].

3.3.5.1. Epidemiology

NODAT has been reported to occur in 2 to 53% of all solid organ transplant recipients [111, 113]. It has been reported to occur in 4 to 25% of renal, 2.5 to 25% of liver, 4 to 40% of heart, and 30 to 35% of lung transplant recipients [114-117]. In hepatitis C virus (HCV)-infected liver recipients, the prevalence of NODAT has been reported to

range between 40 and 60% [115, 116]. Furthermore, it has been reported that African Americans and Hispanics are at increased risk for developing NODAT compared to whites [118]. In Europe, the incidence of NODAT significantly increases according to increased survival rates of solid organ transplantations, ranging from 5% of the transplanted population after 6 months to about 20% after 10 years [119]. As mentioned before, in this population, diabetes leads to serious cardiovascular complications and the risk of mortality markedly increasing with disease evolution. Therefore, the treatment for diabetes in these patients must be initiated as soon as possible. To reduce the risk of diabetes incidence in the patients at risk and especially patients who are most vulnerable, new treatment strategies have been developed, with the early discontinuation of corticoids and the optimization of immunosuppressive treatments, including associations of other therapeutic molecules such as mycophenolate mofetil (MMF) or in some cases, drug conversions [119]. Moreover, screening for diabetes and evaluation of risk factors are recommended to all patients before and after transplantation [120].

4. Immunosuppressive agents

Over the past decade, the advances in immunosuppressive therapy have led to dramatic improvements in graft survival. The development of new agents is the focus of the transplant community, as well as, the establishment of regimens that maintain excellent graft survival rates with less toxicity including infection, nephrotoxicity, malignancy and cosmetic effects [121]. In immunosuppression, the agents commonly employed, include glucocorticoids (GCs), such as dexamethasone, calcineurin inhibitors, such as CsA and FK506. Recently, some potent immunosuppressive drugs became available, such as MMF and the mTOR inhibitors, such as SRL and everolimus. They have allowed the application of new protocols in order to minimize the use of calcineurin inhibitors or steroids due to their diabetogenicity [122]. Despite their desired action on the immune system, these therapies have adverse effects, many being detrimental to graft and even patient long-term survival.

Whereas GCs, CsA and FK506 have been the major responsible for affecting glucose homeostasis after solid organ transplantation, mTOR inhibitors are associated with

hyperlipidemia [123]. In both situations, hyperglycemia occurs because of the imbalance between insulin production and the target tissue insulin demands [124]. NODAT has gained widespread attention due to the micro and macro-vascular complications associated, that increase the morbidity and mortality of patients receiving solid organs [125].

4.1. Glucocorticoids

Ingle in 1941, made the first description of hyperglycemia associated with GCs treatment [126]. However, the benefits of glucocorticoid therapy support its use in maintaining immunosuppressive medication regimens, despite the appearance of NODAT, hyperlipidemia, and other pleiotropic effects of exogenous GCs use in newly transplanted patients. It is known that GCs, such as dexamethasone, promote hepatic gluconeogenesis, degradation of proteins to free amino acids in muscle, and lipolysis [127]. These drugs are also responsible for the decrease in peripheral insulin sensitivity and inhibiting the pancreatic insulin production and secretion by beta-cells [128]. However, there is still not a clear understanding of the mechanisms by which glucocorticoids decrease insulin-mediated glucose uptake. GCs are believed to be the most common cause of drug-induced diabetes *mellitus* [129], however patients presenting decreased insulin secretory reserve before the transplant are much more likely to develop diabetes [130]. Human studies show that GCs are also involved in the alteration of lipid metabolism and the activity of several key enzymes: increased activity of ACC, FAS and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and decreased activity of lipoprotein lipase (LPL) [131]. It has also been observed, the increased hepatic VLDL synthesis and the down-regulation of low density lipoprotein (LDL) receptor activity results in increased VLDL, cholesterol, and triglyceride levels and decreased high density lipoprotein (HDL) levels [130, 132].

4.2. Calcineurin inhibitors

Calcineurin inhibitors have improved the outcome of organ transplants; however, their widespread therapeutic use is restricted due to a number of side effects shared by

these drugs [133]. These agents act against the T-cell activator protein, calcineurin, inhibiting T cell activation and cytokine gene expression. They mediate their immunosuppressive action by interrupting Ca^{2+}/calmodulin - calcineurin signalling pathways in T lymphocytes, thereby blocking antigen-stimulated expression of the interleukin (IL) -2 gene, a growth factor that is critical for T cell proliferation [134, 135]. Calcineurin regulates T-cell transcription factors but also other transcription factors, intracellular inositol 1,4,5-triphosphate receptor (IP3R) [136, 137], and the actin cytoskeleton of kidney podocytes [138]. These drugs have been shown to impair insulin secretion [139, 140], decrease insulin content of the beta-cell [141, 142] and impair insulin transcription [143, 144] although their primary mechanisms of action still remain unexplained. Lawrence et al. (2002) show that in insulin-secreting cells (in vitro study), calcineurin is involved in the stimulation of insulin gene transcription through the activation of the transcription nuclear factor of activated T-cells (NFAT) by its dephosphorylation [143].

Indeed, mice deficient in calcineurin B1 develop diabetes *mellitus* during aging due to insufficient insulin production, while transgenic expression of constitutively active calcineurin/NFAT protects against diabetes *mellitus* [145]. Other studies have suggested that calcineurin may support anti- as well as pro-apoptotic events in the cell [146, 147]. Furthermore, opposing results of transient versus sustained calcineurin inhibition in the beta-cell have also been reported [148] and, collectively, these studies point out that calcineurin controls a number of beta-cell functions subjected to tight regulation. Furthermore, long-term treatment with calcineurin inhibitors is associated with toxic effects, which have a negative impact in the patient's long-term outcome [149]. In addition, both experimental and clinical studies have suggested that these drugs are associated with increasing risk for developing NODAT as mentioned earlier [150, 151]. The individual effects of these agents in clinical studies are difficult to interpret, because concomitant administration of steroids almost always occurs as a confounding factor, which is why regimens minimizing glucocorticoid doses have engendered increased interest. [152]. Throughout this thesis, special attention will be given to the calcineurin inhibitor CsA, and the m-TOR inhibitor SRL, with particular attention to their effects on whole body glucose and lipid metabolism.

4.2.1. Cyclosporin A

Cyclosporin A - CsA (Figure 12) is a hydrophobic cyclic polypeptide consisting of 11 amino acids. It was first described in 1869 from fungi species *Trichoderma polysporum* and *Cylindrocarpo lucidum* [153]. CsA is presently commercially manufactured from the fungi culture *Tolypocladium inflatum* [154]. With high imnunossupressor activity it is an important drug that has been used for more than 20 years in post-transplant recovery. It has been one of the most important drugs in causing the increase in survival rate for post-transplant patients. It is also highly used in therapy for auto-immune deseases [155]

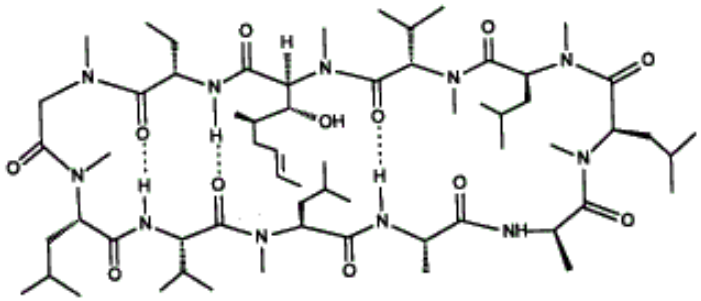

Figure 12 - Structure of Cyclosporin A. Molecular formula: C62H111 N11O12. Molecular weight: 1202.61 g/mol.

4.2.2. Metabolism

Due to the hydrophobic nature of this molecule, cyclosporin A can easily be widespread in the organs and is quickly distributed in the plasma as well as in tissue deposits [156]. It is noteworthy that 90% of the circulating CsA binds to plasmatic proteins and 10% to granulocytes and lymphocytes [157]. More than 70% of the administered cyclosporin A is metabolized by the liver and excreted in the bile, faeces and as much as 10% of its metabolites can be eliminated in the urine [158]. In the human this drug is absorbed in the small intestine and its half-life is between 6 and 9 hours [158].

4.2.3. Mechanisms

Although the molecular events aren't completely understood, there is evidence that suggests that CsA blocks an initial stage in the T lymphocytes activation in addition to inhibiting the production of IL-2 and others lymphokines [158]. Cyclosporin A is able to bind nuclear receptors inhibiting the genetic transcription that encodes the polypeptides segregated by fibroblasts, endothelial cells, monocytes and macrophages [159]. Another theory proposed for the mechanisms of action of CsA, is based on its influence over the cytoplasmic calcium, which is essential for normal cell function [160]. This hypothesis suggests that CsA binds to cytoplasmatic proteins proportionally to its suppressor response [161]. Furthermore, CsA acts over the immunocompetent T lymphocytes, being highly specific for this cells, it also interferes with the initial stages of the cell cycle, affecting the proliferation of lymphoid cells in the early stages of mitosis.[162]. In addition, CsA acts at several stages of an immunologic response to an antigen, selectively inhibiting specific leukocyte functions, restricting the clonal expansion or the functional activation of cell lines [163].

4.3. Sirolimus

Sirolimus - SRL or rapamycin - RAPA (Figure 13) is a macrocyclic lactone isolated from *Streptomyces hygroscopicus.* It belongs to a novel class of immunosuppressants that inhibit mTOR, a key serine-threonine kinase involved in regulation of cell growth and proliferation [164, 165].

In preclinical studies, sirolimus has been shown to be as effective as CsA in maintaining survival of renal and cardiac allografts, without causing nephrotoxicity [166, 167]. Moreover, phase 2 and 3 multicenter clinical trials have demonstrated that sirolimus administered in combination with CsA and steroids (as compared with a control regimen of CsA and steroids) for 2 to 4 months after transplantation, resulted in improvements in long-term patient and graft survival, biopsy-confirmed preservation of renal parenchyma, sustained improvement in renal function and blood pressure, improved quality of life, and less malignancy [168, 169].

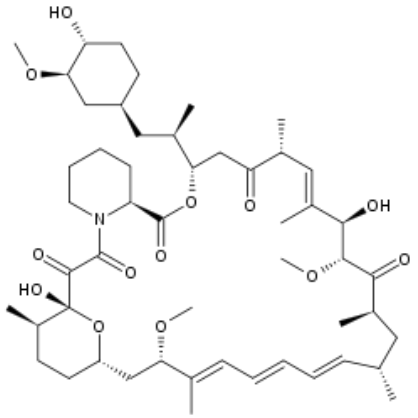

Figure 13 - Structure of Sirolimus. Molecular formula: C51H79N1O13**.** Molecular weight: 914,172 g/mol

4.3.1. Mechanisms

Whereas CsA achieves its effects principally by blocking calcineurin and thereby inhibiting IL-2 production, sirolimus reduces T-lymphocyte activation at a later stage in the cell cycle, by inhibiting the IL-2-mediated signal transduction pathway [170, 135]. Sirolimus effects result from the binding to the immunophilin FK506 binding protein (FKBP12). The creation of a binary complex of SRL and FKBP12 interacts with the SRL binding domain (FKBP-rapamycin-binding, FRB) and thus inactivating a serine-threonine kinase termed the mammalian target of SRL or mTOR, which is known to control proteins that regulate mRNA translation initiation and G1 progression [171]. mTOR is an integrator of multiple signals receiving input from insulin, growth factors, amino acids, and energy to signal to downstream targets and adjust cell growth and proliferation as well as metabolic homeostasis [172]. mTOR also phosphorylates downstream targets, namely, ribosomal protein S6 kinases (S6K) 1 and 2 and the eukaryotic eIF-4E binding protein (4E-BP1) [173]**.** Thus, SRL, an mTOR inhibitor, leads to translational arrest by regulating S6K-1 and 4E-BP1. This way, the immunosuppressive action of SRL is due to the inhibition of T-cell activation at a later stage of the cell cycle, G1, and inhibition of S6K-1 (Figure 14) [174, 175].

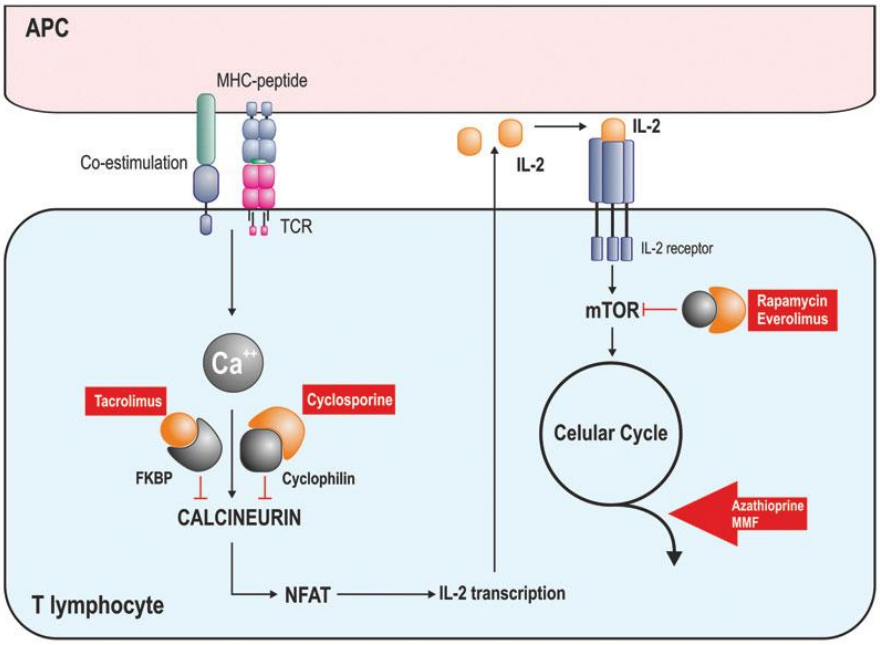

Figure 14 - Inhibition of the immune system by immunosuppressive drugs. On the left: CNIs cyclosporin A/tacrolimus inhibit the Ca_2 dependent processes of IL-2 and other lymphokine synthesis by T-cells during the G0 to G1 transition of the cell cycle**.** On the right: mTOR inhibitors sirolimus/everolimus block Ca_2-independent events durind G1 phase, including transduction of the seconds signals delivered by IL-2, IL-4 or IL-6. Abbreviation: MHC, Major histocompatibility complex; TCR, T-cell receptor; FKBP, FK506 binding protein; NFAT, nuclear factor of activated T-cells; IL-2, Interleukin-2; mTOR, mammalian target of rapamicin; MMF, mycophenolate mofetil. Adapted from ref. [176].

The effects of SRL on insulin action and secretion are being debated, and data are conflicting. In fact, in skeletal muscle cells, long-term exposure to SRL has been shown to decrease insulin-dependent glucose uptake, glycogen synthesis and increase fatty acid oxidation [177]. In addition, while there are studies showing that SRL decreases insulin-mediated glucose uptake and insulin signalling in 3T3-L1 and 3T3-F442A adipocytes [178], others report decreased insulin resistance induced by hyperinsulinemia and partially improve insulin-dependent glucose transport in 3T3-L1 cells [179]. Elevated concentration of glucose was also observed by SRL treatment suggesting that when glucose uptake by cells is not efficient, this leads to more insulin secretion by the pancreas. Sirolimus potentially can worsen insulin resistance, but more studies are required, because data are conflicting [179, 152] The pathogenic mechanisms of IAs are summarized on Table 2.

Table 2 - Drug-induced NODAT: potential pathogenic mechanism(s)

Immunosuppressive agent	Proposed mechanism(s)	Comments
Corticosteroids	• ↓ Peripheral insulin sensitivity • Inhibit pancreatic insulin production and secretion • ↑ Hepatic gluconeogenesis • Promote protein degradation to free amino acids in muscle, lipolysis	• Dose-dependent • Impact of complete withdrawal of chronic low-dose steroids unclear • Potential ↓ NODAT risk in steroid-free regimens
Cyclosporin A	• ↓ insulin secretion (CsA < FK506) • ↓ insulin synthesis • ↓ beta-cell density	• Dose-dependent, • Diabetogenic effect ↑ with ↑ steroid dose*
Sirolimus	• ↑ Peripheral insulin resistance • Impair pancreatic beta-cell response	• Diabetogenicity when use with CNIs

Note: * Demonstrated in some but not all studies.
Abbreviations: CsA, cyclosporin A; CNIs, calcineurin inhibitors; ↑, increate; ↓, decreased [113].

4.4. Side effects

The most common side-effects of these drugs have been: the development of arterial hypertension, vascular lesions (atherosclerosis or arteriosclerosis), nephrotoxicity and neurotoxicity [180]. More rarely headaches, rashes from an allergic reaction, light anemia, pancreatitis and convulsions can also occur. The side effects are normally dose-dependents responding quickly to a dose reduction [181]. Cyclosporin A appears to decrease insulin secretion, by interfering with the cytochrome P-450 system in renal transplant recipients, thereby worsening the potential for hyperglycemia [182], and a decrease in insulin and C peptide secretion in non-transplanted hemodialysis subjects has

also been observed [183]. In animal models, with calcineurin inhibitor treatment, it is possible to observe a decrease in glucokinase activity and reduced insulin gene expression, with resultant decrease in insulin secretion [177, 184, 131].

After glucocorticoids, cyclosporin A is a common cause for the appearance of post-transplant hyperlipidemia and it appears to be dose- and treatment duration-related. Increase in plasma cholesterol with elevation of LDL levels as been demonstrated in studies in nontransplant subjects, receiving cyclosporine A [185]. It has been suggested that CsA inhibits steroid 26-hydroxylase, an important mitochondrial enzyme that enables bile acid synthesis from cholesterol [131]. This leads to an increase in hepatic cholesterol, and down-regulation of the LDL receptor resulting in hypercholesterolemia. Cyclosporin A is carried by LDL particles and can bind to the LDL receptor in the intestines [186]. Increasing LDL cholesterol levels, reduces post-heparin lipolytic activity, and decreases LPL activity, which can be seen in impaired clearance of VLDL and LDL cholesterol [129, 187, 188].

Cardiovascular risk is real for transplant recipients and it could be explained by the pro-oxidant effect of cyclosporin A that accelerates atherosclerosis. The inhibition of calcineurin might also be responsible for hypertension, as it increases vascular tone and systemic vascular resistance [184]. Discontinuation of cyclosporin A is associated with improvement in hyperlipidemia; this effect could be related to improvement in kidney function and concomitant reduction of steroid doses.

As the significant nephrotoxicity, neurotoxicity, and hypertension associated with CsA can be attributed in part to calcineurin blockade [185, 186], SRL would be expected to have a different toxicity profile. On the other hand, this successful and most recent drug has a serious side effect, which is to cause hyperlipidemia in renal, pancreatic, and liver transplant patients [187-189]. Sirolimus-associated dyslipidemia has been reported in 49% of liver transplant patients [190, 191] and in about 40% of renal transplant patients [192].

When SRL is administrated to animals like guinea pigs, there is an increase in triglyceride levels, increased VLDL and small dense LDL, and higher glucose and circulating free fatty acid levels have also been observed [184]. SRL induces or exacerbates hyperlipidemia in a reproducible, reversible and dose-dependent manner in

some renal transplant recipients. The mechanisms that cause hypertriglyceridemia due to sirolimus treatment, might be increased hormone sensitive lipase and decreased LPL activity secondary to elevated apolipoprotein C-III levels [123]. This inhibitor of LPL [178] reduces the catabolism of apolipoprotein B100-containing lipoproteins [193] resulting in an increased free fatty acid pool [194]. Increased hepatic synthesis and delayed clearance of triglyceride-rich lipoproteins have been implicated as potential mechanisms of sirolimus-induced hypertriglyceridemia [123]. SRL also appears to alter insulin signalling in adipose tissue by increasing lipase activity and/or decreasing lipoprotein lipase activity, resulting in increased hepatic synthesis of triglycerides, increased secretion of VLDL, and increased hypertriglyceridemia [129]. Overall, the benefits of sirolimus supersede its dyslipidemic effects, because it leads to use of calcineurin inhibitor-sparing regimens, and therefore a lower incidence of nephrotoxicity [152].

4.5. Combination SRL/CsA

The synergistic combination of SRL and CsA may offer a unique immunosuppressive strategy for organ transplantation. Combinations of the two drugs produced synergistic prolongation of heart or kidney allograft survival at SRL/CsA ratios ranging from 1:12.5 to 1:200 [196]. The synergistic interaction between SRL and CsA may also be related to their sequential molecular mechanisms of action. As explained above, CsA inhibits the Ca_2 dependent processes of IL-2 and other lymphokine synthesis by T-cells during the G0 to G1 transition of the cell cycle [197]. In Contrast, SRL blocks Ca_2-independent events during G1 phase, including transduction of the seconds signals delivered by IL-2, IL-4 or IL-6 [194].

SRL was initially used in immunosuppressive regimens, thus allowing the minimization or avoidance of CNIs, but despite SRL's little or no nephrotoxicity by itself [114], it potentiates CsA's nephrotoxicity [187]. Podder et al. suggested that this impairment of renal function is due to a pharmacokinetic interaction of SRL that greatly increases the CsA concentration in whole blood and, particularly, on kidney tissue [127]. CNIs' associated toxicities are related to their blood and tissue concentrations. However, drug levels are unpredictable, due to intraindividual and interindividual differences in drug

pharmacokinetics, including hepatic drug metabolizing activity and drug absorption in the small intestine [131].

5. Aim of the study

The overall aim of this study was to elucidate the molecular mechanisms of action of cyclosporin A and sirolimus, *in vivo*, in a rat model.

The following aspects were addressed:

1. The effects of 6 weeks CsA and SRL in *in vivo* treatments on body weight, glucose tolerance, whole body glucose, insulin and lipid levels.

2. The effects of CsA and SRL on glucose transport in isolated rat adipocytes after 6 weeks of treatments.

3. Gene and protein expression in liver, muscle and isolated adipocytes.

a) IRS-1, Glut4 and Glut1 expression in liver, muscle and fat.

b) IL-6, TNFα and Adiponectin gene expression in fat.

c) DGAT1 and PGC1α expression in liver, muscle and fat.

d) FoxO1, SREBP-1, ChREBP, ACC1: gene and protein expression.

Chapter II. Materials & Methods

II – Materials & Methods

1. In vivo study

1.1. Animals housing

Male Wistar rats (Charles River Lab. Inc, Barcelona, Spain) were housed two animals per cage, kept at a constant temperature (21°C) and light (06:30–18:30 h) / dark (18:30–06.30 h) cycle. They were given standard laboratory rat chow (IPM-R20, Letica, Barcelona, Spain) and free access to tap water. Body weight was measured weekly (Monday 09:00 h). All animal care and experimental procedures were conducted according to the guidelines of the National and European Communities Council Directive (86/609/EEC).

1.2. Treatments

An initial study was performed on rats fed a standard diet. In this experiment, 10 week-old animals (316,4 ± 2,5 g) were randomly divided into three groups: vehicle group (30% orange juice in sterile water); a cyclosporin A group - 5 $mg \cdot kg^{-1} \cdot day^{-1}$ of Sandimun Neoral® and a sirolimus group - 1$mg \cdot kg^{-1} \cdot day^{-1}$ of Rapamune® dissolved in orange juice. Animals were treated during 6 weeks with daily oral gavage injections, of either vehicle, CsA or SRL (Figure 15). Doses of CsA and SRL were chosen according to the blood concentrations observed in clinical practice in patients after organ-transplantation: the recommended therapeutic windows of CsA in blood are 200-400 ng ml^{-1} for 2 month and 100-200 ng ml^{-1} thereafter. For SRL, doses are adjusted to achieve a level of 30 ng ml^{-1} for the first 2 months and 15 ng ml^{-1} thereafter. [198].

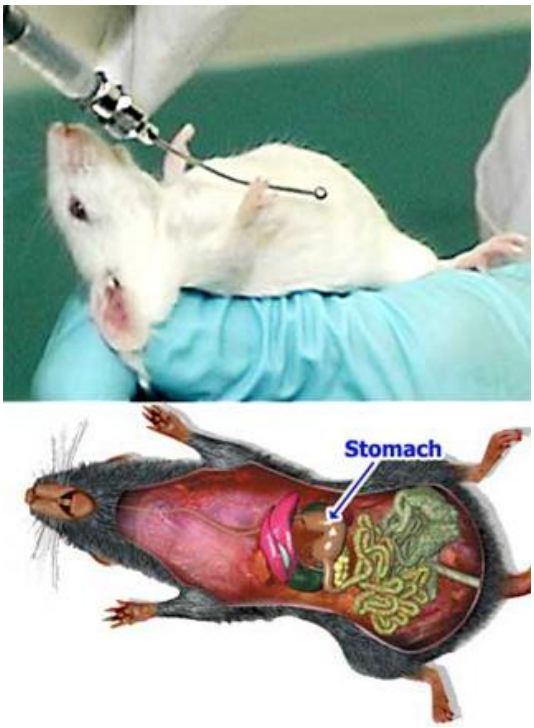

Figure 15 - Procedure for oral gavage.

1.3. Glucose tolerance test (GTT)

A GTT was performed in fasted rats, 3 days before sacrifice. The last injection of vehicle, Cyclosporin A or Sirolimus was administered 16 h before the GTT. Rats were food-deprived for 16 h (17.30–09.30 h), and a glucose load of 2 $g \cdot kg^{-1}$ was administered i.p. The glucose levels were measured using a glucometer (AccuChek Active, Roche Diagnostics Inc., Indianapolis, IN, USA). The blood was collected, by tail vein bleeding, immediately before (0 min), at 15', 30', 60' and 120' after glucose injection and the area under the curve (AUC) was used to compare differences in the glucose excursion curves among groups.

1.4. Sacrifice

At the end of treatments the rats were anesthetized i.p. with 2 mg Kg^{-1} body weight of a 2:1 (v:v) 50 mg mL^{-1} Ketamine (Ketalar®, Parke-Davis, Pfizer Laboratories Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largatil®, Rhône-Poulenc Rorer,

Vitória laboratories, Amadora, Portugal). Blood samples were immediately collected by venipuncture from the jugular vein in needles with no anticoagulant, for serum sample collection or with appropriate anticoagulant (ethylenediamine tetraacid – EDTA) for plasma samples for further analysis. Glucose, TGs, Total-Cholesterol, HDL and LDL were measured in serum through automatic validated methods and equipments (Hitachi 717 analyser, Roche Diagnostics Inc., Holliston, MA, USA). TGs in liver and muscle were also measured through a Triglyceride Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). Fed insulin levels in blood were determined by ELISA kits (Mercodia, Uppsala, Sweden).

CsA and SRL blood concentrations were assessed by immunoassay using automatic methods (Flex reagent) and equipment (Dimension®RxL, Siemens, Germany). Rats were sacrificed through rapid cervical dislocation. Blood was collected, and tissues were frozen in liquid nitrogen and stored at -80°C for further analyses.

2. Chemicals

Cyclosporin A (Sandimmune Neoral®) was kindly supplied by Novartis Pharma (Lisbon, Portugal), while SRL (Rapamune) was provided by Wyeth Europe Ltd (Berkshire, UK). Collagenase, type II from *Clostridium histolyticum*, and glucose strips were purchased from Roche (Lisbon, Portugal). KHR buffer was prepared with 4% Bovine Serum Albumin (BSA), 140mM Sodium Chloride (NaCl), 4,7 mM Potassium Chloride (KCl), 1,25 mM Magnesium Sulfate ($MgSO_4$), 1,26 mM Calcium Chloride ($CaCl_2$), 5,8 mM Sodium Phosphate (NaH_2PO_4), 200nM adenosine deaminase and 25mM Hepes, all obtained from Sigma Chemical Co. (St. Louise, MO, USA). D-[U-14C] glucose (specific activity, 200-300 mCi/mM) was purchased from Scopus Research BV (Wageningen, The Netherlands). Human insulin, Actrapid, 100 U/ml was a kind gift from Novo Nordisk A/S (Paço de Arcos, Portugal).

High Capacity cDNA Reverve Transcription kit was obtained from Applied Byosistems (Forest City, CA, USA). RNeasy® MiniKit (250) and QIAzol® Lysis Reagent were obtained from QIAGEN Sciences (Germantown, MD, USA); diethyl pyrocarbonate (DEPC) was acquired from AppliChem, (Darmstadt, Germany). Methanol and isopropanol

were obtained from Merck (Darmstadt and Hohenbrunn rispectively, Germany). All primers were obtained from IDT-Integrated DNA Technologies, Inc (Coralville, IA, USA).

RIPA buffer (Radio Immuno Precipitation Assay buffer) was prepared with 20 mM Tris HCl pH 7.4, 25 mM NaCl, 1% NP-40 (Nonidet P-40), 5 mM EDTA, 10 mM Sodium diphosphate ($Na_4P_2O_7$), 10 mM Sodium Fluoride (NaF), 2 mM Sodium Vanadate Na_3VO_4, 10 μg ml^{-1} Aprotinin from bovine lung, 1 mM Benzamidine and 1 mM Phenylmethylsulfonyl fluoride (PMSF), (Sigma-Aldrich, St. Louise, MO, USA).

Pierce® BCA Protein Assay Kit (bicinchoninic acid) was obtained from Termo Scientific (Rockford, IL, USA). 30% Acrylamide/BisSolution 19:1 (5% c) was obtained from Bio-Rad Laboratories, Inc (Hercules, CA, USA) and TEMED ("N,N,N',N' Tetramethylethylene-diamine) from Sigma-Aldrich, Inc (St. Louise, MO, USA); the polyvinylidene difluoride (PVDF) membranes from EMD Millipore Corporation (Billerica, MA, USA). Rabbit anti-FoxO1 antibody, rabbit anti-ACC1 antibody were purchased from Cell Signaling Technology (Danvers, MA, USA) and distributed through Izasa Lisbon (Portugal); mouse anti-SREBP-1(2A4), goat anti-ChREBP (P-13), as well as alkaline phosphatase-linked secondary antibodies goat anti-rabbit, goat anti-mouse and rabbit anti-goat were obtained from Santa Cruz Biotechnology, Inc, (Santa Cruz, CA, USA). Antibody against b-actin was purchased from BioLegend, Inc (San Diego, CA, USA). The enhanced chemifluorescence (ECF) reagent was obtained from GE Healthcare (Carnaxide, Portugal). All other reagents were purchased from Sigma Chemical Co.

3. Glucose uptake

3.1. Adipocyte isolation

After sacrifice, rat epididymal adipose tissue was immediately removed, cut into small pieces and digested with collagenase type II (0,6 mg ml^{-1}) in 6 mM glucose KHR buffer (as described above), pH 7.4, at 37°C with gentle shaking for 30 min. The resulting cell suspension was isolated from the undigested tissue by filtration through a 250 μm nylon mesh and washed four times in medium without glucose (4% BSA, 200 nM adenosine and pH 7.4, adjusted with NaOH). [199].

3.2. Glucose uptake assay

Insulin-stimulated ^{14}C-glucose uptake in isolated rat adipocytes was assessed as previously reported [200]. Briefly, freshly isolated adipocytes were diluted ten times in KHR buffer without glucose (4% BSA, 200nM adenosine and pH 7.4) and 500 µl cell suspension was placed in a shaking water-bath (90 rpm). Adipocytes were incubated at 37ºC for a further 10 min with or without 1 mU/ml human insulin (10nM) before the addition of D-[U-^{14}C] glucose (0.30 mCi/L, final conc. 860 nM) for another 30 min (Figure 16). Cell suspension was then transferred to pre-chilled tubes, containing silicone oil, allowing the cells to be separated from the buffer by centrifugation for 5 min at 3000 x g. Cell-associated radioactivity was analyzed by liquid scintillation counting in a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer Life, Shelton, CT, USA), allowing us to determinate the rate of trans-membrane glucose transport. Experiments were performed in triplicates. [201, 202]. Finally, the rate of trans-membrane glucose transport was calculated according to the following formula: cellular clearance of medium glucose = (c.p.m. cells x volume)/(c.p.m. medium x cell number x time) [203].

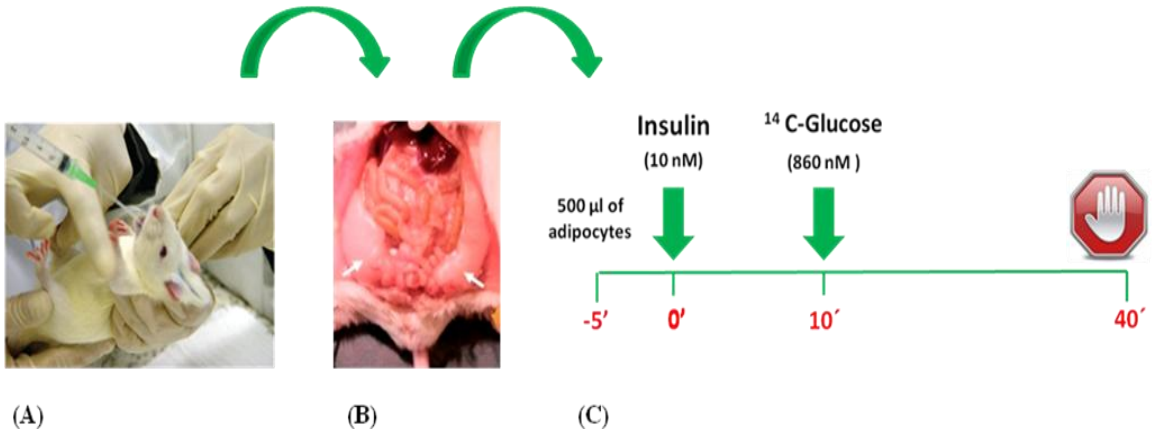

Figure 16 - Insulin-stimulated ^{14}C-glucose uptake in rat adipocytes. (A) In vivo treatments by oral gavage (is not injections) of either vehicle, CsA or SRL. (B) Rats were sacrificed and apididymal fat collected. (C) Freshly isolated adipocytes were obtained by collagenase type II digestion and incubated with or without 10 nM insulin for 10 min. followed by D-[U-14C] glucose (0.30 mCi/L, 860 nM) for 30 min.The rate of insulin-stimulated glucose uptake was then calculated. Experiments were performed in triplicate.

3.3. Triglycerides extraction

After obtaining the final cell solution (1:10) 500µl of cell suspension was pipetted into glass tubes with triglyceride extraction solution containing a 2,8 µl stock solution (780 ml isopropanol, 200 ml n-heptane, 20 ml H_2SO_4 0,5M), 1,8 ml heptane, 1ml H_2O. The tubes were shaken vigorously and left for 24h at room temperature before centrifuging for 5 min at 3000 x g. The upper phase was placed in previously weighed vials and allowed to dry in the hood. Afterwards the final weight obtained represents the triglyceride mass in 500 µl cell suspension. Experiments were performed in triplicates.

3.4. Measurement of adipocyte diameter

The average cell diameter was measured in isolated adipocytes using a B1 series microscope (System Microscopes-Motic) and a 40X ocular provided with an internal ruler. 150-200µl of cell solution (1:10) was placed on previously fixed slides with Silicon Oil and heated to 100 ° C for 1 hour. The diameter of 100 consecutive cells from each subject was measured and entered into a program to calculate individual fat cell diameter and size [204].

Finally, the cell weight obtained was introduced into the following formula in order to calculate the number of cells per 500µl of solution:

$$cell\ number = \frac{2.35 \times \text{triglyceride mass}}{\text{cell weight}} \times 10^6$$

4. Real-time reverse transcription by polymerase chain reaction

Liver, muscle and fat tissues were used for mRNA quantification by real-time reverse transcription polymerase chain reaction (real-time RT-PCR), to analyze the followed genes: forkhead box O1 (FoxO1); sterol regulatory element-binding transcription factor 1 (SREBF1); carbohydrate response element-binding protein (ChREBP); diacylglycerol acyltrasferase 1 (DGAT1); acetyl-CoA carboxylase 1 (ACC1); peroxisome proliferator-activated receptor γ coactivator 1 (PGC1); insulin receptor substrate-1 (IRS-1);

glucose transporter type 4 (Glut4); glucose transporter type 1 (Glut1); interleukin-6 (IL-6); tumor necrosis factor alpha (TNFα); and adiponectin. RNA was isolated with the RNeasy mini-kit and the cDNA was synthesized using a High Capacity cDNA Reverse Transcriptase kit. Gene expression was analyzed using an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad laboratories, Hercules, CA, USA). Gene expression was normalized using the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Primers sequences are listed on Table 3.

Table 3 - Primer sequences for targeted cDNAs

Primers name	5'-3' sequence	Source
ACC1	F: AAGGCTATGTGAAGGATGTGG R: GAGGTTAGGGAAGTCATCTGC	Rat
AdipoQ	F: AAGTCTGGCTCCAAGTGTATG R: AGCAATACAATCAACCTCTCAAAC	Rat
DGAT1	F: GACAGCGGTTTCAGCAATTAC R: GGGTCCTTCAGAAACAGAGAC	Rat
FoxO1	F: GGATAAGGGCGACAGCAACA R: TGAGCATCCACCAAGAACT	Rat
GAPDH	F: AACGACCCCTTCATTGACC R: CACGACATACTCAGCACCAG	Rat
Glut1	F: TGCAGTTCGGCTATAACACC R: CCCACAGAGAAGGAACCAATC	Rat
Glut4	F: CGTCATTGGCATTCTGGTTG R: CTTTAGACTCTTTCGGGCAGG	Rat
IL-6	F: CTGGAGTTCCGTTTCTACCTG R: CCTTCTGTGACTCTAACTTCTCC	Rat
IRS-1	F: ACGCTCCAGTGAGGATTTAAG R: CCTGGTTGTGAATCGTGAAAG	Rat
MLXIPL (ChREBP)	F: CTTATGTTGGCAATGCTG R: GGCGATAATTGGTGAAGA	Rat
PGC1	F: TGTTCCCGATCACCATATTCC R: CTTCATAGCTGTCATACCTGGG	Rat
SREBF1	F: CGCTACCGTTCCTCTATCAATG R: TCAGCGTTTCTACCACTTCAG	Rat
TNFα	F: CTTCTCATTCCTGCTCGTGG R: TGATCTGAGTGTGAGGGTCTG	Rat

Abbreviations: ACC1, Acetyl-CoA carboxylase; AdipoQ, Adiponectin; DGAT1, Diacylglycerol acyltrasferase1; FoxO1, Forkhead box O1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; Glut-1, Glucose transporter type-1; Glut-4, Glucose transporter type; IL-6, Interleukin; IRS-1, Insulin receptor substrate; ChREBP, Carbohydrate response element-binding protein; PGC1, Peroxisome proliferator-activated receptor γ coactivator; SREBF1, Sterol regulatory element-binding transcription factor 1; TNFα, Tumor necrosis factor alpha.

4.1. RNA extraction

Total RNA was isolated from liver, muscle and perirenal fat cells according to the manufacturer's instructions (Figure 17), to later determine the transcription levels of different molecules in the cell. Briefly, ≤100 mg fatty tissue and ≤50 mg of liver and muscle were disrupted and homogenized in 1 ml QIAzol® lysis reagent using an ULTRA-TURRAX® T 25 basic homogenizer. After addition of 200µl of chloroform, samples were vortexed, incubated for 2min at room temperature and centrifuged at 12,000g, for 15min, at 4◦C. The aqueous phase containing RNA was transferred to a new tube, adding 1 volume of 70% ethanol. All content was transferred to an RNeasy column, centrifuged for 15 sec at ≥8000xg and the flow-through was discarded (performed twice). Last steps include RW1 and RPE buffer addition followed by repeated centrifugations at ≥8000 x g.

Finally, the RNeasy column was placed in new 2 ml tube, 30-50 µl RNase-free water (previously heated at 50-60°C) was added and centrifuged for 1 min at ≥8000 x g. RNA concentration was then determined by OD260 measurement using a Nanodrop spectrophotometer (Wilmington, DE, USA). RNA was stored at −80◦C.

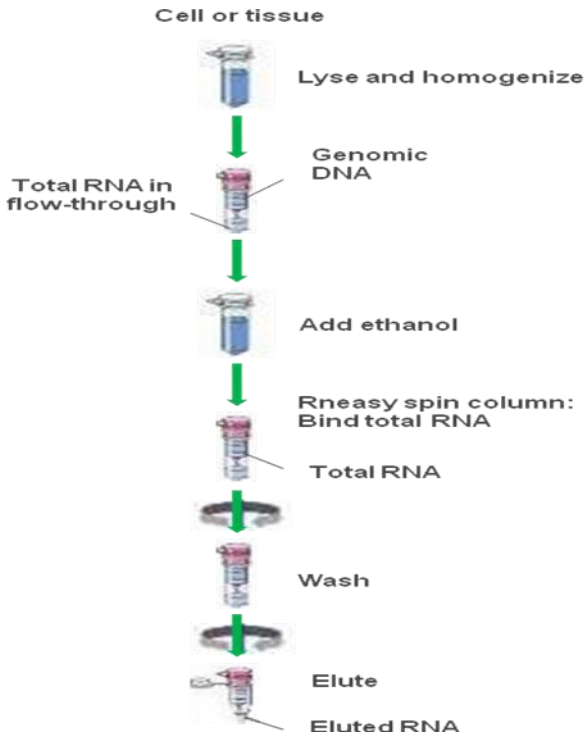

Figure 17 - RNeasy mini kit procedure

4.2. Real-time RT-PCR

In the real-time RT-PCR technique total RNA is initially converted into cDNA, by reverse transcription. A specific sequence of the cDNA (the gene in study) is amplified using specific primers, and the inclusion of a fluorescent dye allows the detection of the reaction progress in real time. Finally, real-time RT-PCR allows the measurement of gene amplification, important to compare the amount of gene expression in the cell, in a control or treatment situation.

Two micrograms of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription (RT), from Applied Byosistems. Briefly, 2µl of 10X RT Buffer, 0.8µl of 25X dNTP Mix, 2µl of 10X RT random primers, 1µl of MultiscribeTM Reverse Transcriptase and 4.2µl of nuclease free H20 were added to 10µl of RNA (2µg) sample. A protocol for cDNA synthesis was run on all samples (10min at 25°C, 120min at 37°C, 5min at 85°C and then put on hold at 4°C). After the cDNA synthesis, the samples were diluted with RNase-free water up to a volume of 200µl and concentration of 5ng/µl.

Real-time RT-PCR was performed in a 10µl volume containing 2.5µl cDNA (12,5ng), 5µl 2X SYBR Green Fastmix, 0,03µl of each primer (100 µM) and 2,44µl of H2O PCR grade. The amplification conditions are reported on Table 4.

Table 4 - Real-Time PCR amplification conditions

		45 repeats			81 repeats
	denaturation	denaturation	annealing	extention	melting curve
Temperature	95°C	95°C	60°C	72°C	55°C
Time	3 min	10 sec	30 sec	30 sec	10 sec

5. Western Blot analyses

5.1. Cell lysate preparation

Ten g of liver and muscle were weighed and homogenized in the 400 µl in ice-cold RIPA buffer. Cell lysates were homogenized three times, during 5 sec, at 13500 rpm using an ULTRA-TURRAX® T 25 basic, IKA®-Werke (Staufen, Germany) homogenizer, to disrupt cells. Following, samples were centrifuged at 14 000 g at 4°C for 10 min and the protein concentration in the supernatant was assessed. Protein concentration was determined using the bicinchoninic acid (BCA) method. Cell lysates were denatured at 95ºC, for 5min, in sample buffer (0.5 M Tris HCl pH 6.8; 10% (w/v) SDS; 0.6 M DTT; 30% (v/v) glycerol and 0.01% bromophenol blue).

5.2. Protein quantification by the bicinchonininc acid method

The bicinchonininc acid (BCA) method is a copper-based protein assay used for protein quantification, sustained on the well-known "biuret reaction", whereby peptides containing three or more amino acid residues form a colored chelate complex with Cu2+, in an alkaline environment containing sodium potassium tartrate. The BCA method involves two different step reactions: the biuret reaction, whose blue color results from the reduction of Cu+2 to Cu+1 by proteins; and the BCA chelation with Cu+1, resulting in an intense purple color (Figure 18). The purple colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. The purple product absorbs strongly at 540-570nm, which absorption can be read in a spectrophotometer. Since the production of copper in this assay is a function of protein concentration and incubation time, the protein content of unknown samples may be determined spectrophotometrically by comparison with known protein standards. Indeed, at the same time, the absorption of bovine serum albumin (BSA) of increasing concentrations between 12.5 µg mL^{-1} and 800µg mL^{-1} was determined and used as a standard linear curve to determine protein concentration. Equal amounts of diluted protein samples (1:9) and water or BSA dilutions and the sample buffer RIPA in a final volume of 50µL were placed in a 96 multi-well plate. Then, 200µL of BCA reagent was added to the wells and the plate was incubated in the dark for 30min, at 37ºC. After incubation, the absorption was measured in an automatic microplate reader (SLT, Austria) at 570nm. This method is not affected by a range of

detergents and denaturing agents such as urea and guanidinium chloride, although it is more sensitive to the presence of reducing sugars [205].

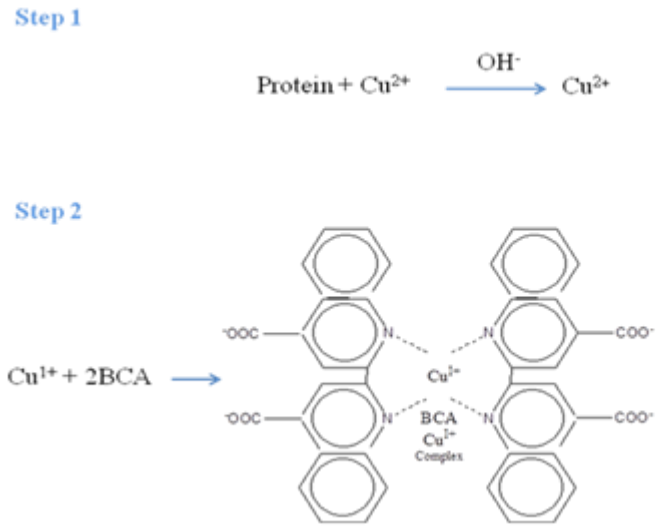

Figure 18 - Schematic reaction for the bicinchoninic acid (BCA)-containing protein assay.

5.3. SDS-PAGE, PVDF transfer and WB analysis

Western Blot (WB) analyses was used to determine proteins levels inside of the cell, giving us information about different protein expression levels, as well as, its activation (phosphorylated proteins).

Equal amount of proteins were loaded in the gel: 20 or 40µg depending on the quantity needed to observe the protein band. Proteins were separated by electrophoresis on a 7,5% (v/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane, then covered with ponceau staining (Sigma) to confirm equal amount of protein in each well, subsequently was blocked with Tris buffer with 0.01 % of Tween 20 (TBS-T, pH 7.4) containing 5% dry milk for 1h at room temperature. Later membranes were incubated overnight at 4ºC with rabbit anti-FoxO1 antibody (dilution 1:1000), rabbit anti-ACC1 antibody (dilution 1:1000); and mouse anti-SREBP-1 antibody (dilution 1:1000), goat anti-ChREBP (dilution 1:1000), according to manufacturer instructions. Mouse β-actin (dilution 1:1000) or anti-Akt2/PKB-β were used as loading controls.

After incubation, membranes were washed three times for 15min with 0.1% TBS-T and incubated for 1h at room temperature with either alkaline phosphatase-conjugated anti-rabbit antibody (1:5000), alkaline phosphatase-conjugated anti-mouse antibody (1:5000) or alkaline phosphatase-conjugated anti-goat antibody (1:5000). The immune complexes were detected by membrane exposure to the ECF reagent, during 4 to 6min, followed by scanning for blue excited fluorescence on a VersaDoc™ Imaging System, Bio-Rad (Bio-Rad Laboratories, Amadora, Portugal). The generated signals were quantified using Quantity One™ Software.

6. Statistical analysis

Results are given as mean ± standard error of the mean (SEM) using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA, USA). Statistical analyses using the Student's *t*-test were performed when two groups were considered. For multiple group comparisons, the One-Way ANOVA test, followed by the *post hoc* Bonferroni´s Multiple Comparison was used. Differences were considered significant when $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$.

Chapter III. Results

III - Results

1. Growth curves

Body weight was weekly monitored throughout the study. Growth curves in male Wistar rats show that controls/vehicle treated animals have normal body growth from the first week (316,3 ± 3,9 g) to the sixth week (395,3 ± 7,0 g) of treatment, as well as the cyclosporin A group (314,8 ± 5,4 to 407,7 ± 7,8 g). Conversely, the SRL treated animals were significantly lighter starting at week 5 (353,3 ± 5,7 vs 379,0 ± 6,4 g; p <0,05) and trough week 6 (361,7 ± 5,8 vs 395,3 ± 7,0 g; p <0,001), as compared to the vehicle treated group. Furthermore, body weights of SRL treated rats are lower already at the 4th (354,0 ± 5,1 vs 383,2 ± 6,0 g; p<0,01), 5th (353,3 ± 5,7 vs 395,5 ± 6,4 g; p<0,001) and 6th weeks (361,7 ± 5,8 vs 407,7 ± 7,8 g; p<0,001), in comparison to the CsA treated group (Figure 19).

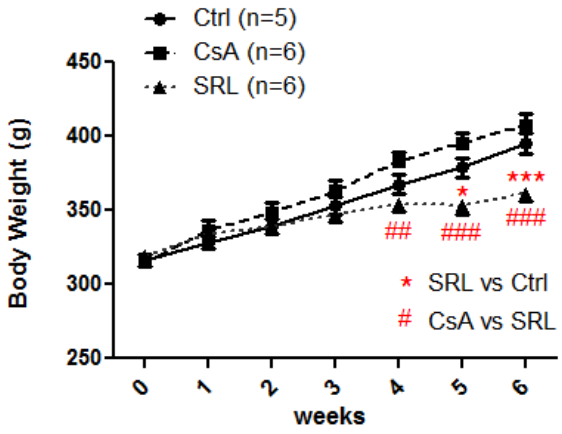

Figure 19 - Growth curves for the Control (Ctrl) Cyclosporin A (CsA) and Sirolims (SRL) groups, in male Wistar rats from 10 to 16 weeks of age. Data are shown as mean ± S.E.M. Two way ANOVA, **p* <0.05 or ****p* <0.001 vs Ctrl; ## *p*<0,01 or ### *p*<0,001 vs SRL

2. Serum glucose and insulin

Glucose levels were determined in serum from fed and fasted rats. Rat fed glucose levels were significantly higher after 6 weeks of SRL treatment, as compared to the vehicle (254,5 ± 18,1 vs 163,4 ± 13,4 mg/dl; p<0,01) and the CsA treated groups (254,5 ± 18,1 vs 180,2 ± 19,7 mg/dl; p<0,05) (Figure 20B). No significant differences were observed in either fasted glucose (Figure 20A) or fed insulin levels (Figure 20C), in these animals. Fasted insulin levels were not measured.

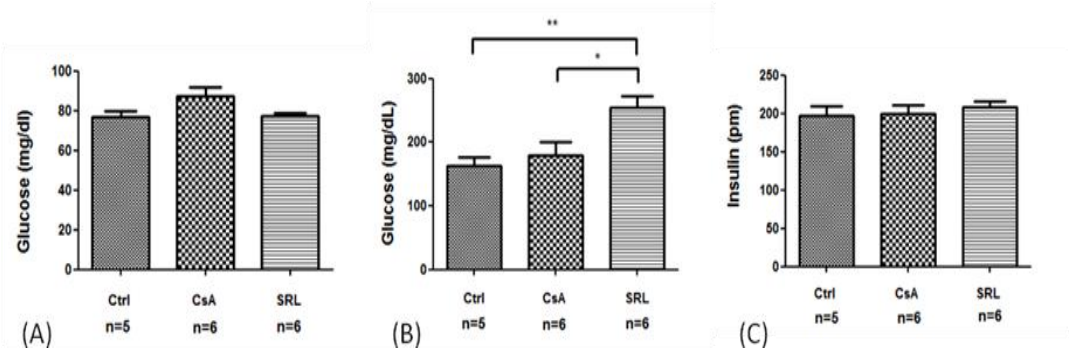

Figure 20 - Serum glucose and insulin levels in Wistar rats after 6 weeks of cyclosporin A and sirolimus treatments. (A) Fasted glucose; (B) Fed glucose; (C) Fed insulin. Serum glucose and insulin were determined as reported in Matherials & Methods. Data are shown as mean ± S.E.M. Ctrl, control; CsA, cyclosporin A; SRL, sirolimus. t-test, *p <0.05 or **p <0.01.

3. Glucose tolerance test

The fasted glucose levels, before a glucose tolerance test, presented no significant differences either in cyclosporin A (88,0 ± 5,5 vs 73,6 ± 4,6 mg/dl) or the sirolimus treated rats (77,4 ± 2,0 vs 73,6 ± 4,6 mg/dl), compared to vehicle treatment, as shown above (Figure 20A). However, glucose levels were significantly higher for both the CsA (336,0 ± 50,9 vs 165,6 ± 10,1 mg/dl; p=0,001) and the SRL treated animals (311,6 ± 44,2 vs 165,6 ± 10,1 mg/dl; p=0,001) at the 15' time point compared to vehicle. These glucose values persisted significantly elevated until the 60' min time point for the SRL treated animals, indicating that these animals are glucose intolerant, while the glucose excursion for the CsA treated group returned to normal after 30 min and was no different from to the vehicle treated group, as shown on Figure 21A. The results are also presented as the AUC histogram showing the significant difference between the glucose curves for both the SRL (30032 ± 2201 vs 17856 ± 791,0; p<0,001) and the CsA (25491 ± 3344 vs 17856 ± 791,0; p=0,0570) treated groups, compared to vehicle, demonstrating an impaired glucose excursion during a GTT (Figure 21B).

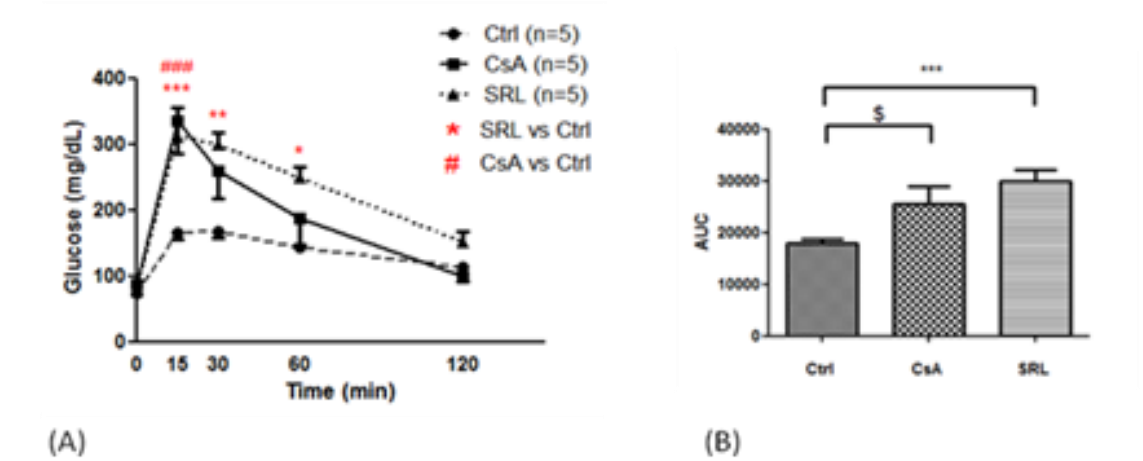

Figure 21 - Glucose tolerance tests (GTT) were performed in 16- week old Wistar rats after 6 weeks of cyclosporin A and sirolimus treatments. A glucose load of 2 g·kg^{-1} (or 2 mg g^{-1}) was administered i.p after an overnight 16h fast. Glucose levels were measured at 0', 15', 30', 60' and 120', as described in Materials & Methods. (A) Glucose levels during a GTT: Ctrl vs. CsA 5 mg/kg/day vs. SRL 1mg/kg/day**.** (B) Area Under the Curve. Data are shown as mean ±S.E.M. ●**,** Vehicle (Ctrl), ■ cylosporin A (CsA) and ▲ sirolimus (SRL) treatments; Two way ANOVA and t-test, as appropriate, **p* <0.05, ***p* <0.01 or ****p* <0.001; $ p=0,0570.

4. Lipid profile

Cholesterol and trygliceride levels were analyzed in order to study the in vivo effects of CsA and SRL treatments on lipid metabolism by assessing fat concentration/storage in blood and tissues.

4.1. Serum cholesterol levels

Serum fed cholesterol levels were assessed in the three different groups. As shown in Figure 22A, total cholesterol was significantly elevated in either SRL (61,7 ± 2,5 vs 47,4 ± 1,6 mg/dl; p <0,001), or CsA treated groups as compared to vehicle treated animals (52,9 ± 2,0 vs 47,4 ± 1,6 mg/dl; p <0,05). In addition, serum HDL cholesterol (Figure 22B) was significantly elevated in the SRL treated group as compared to vehicle treated animals (35,0 ± 1,0 vs 27,2 ± 1,1 mg/dl; p <0,001), and to the CsA treated group (35,0 ± 1,0 vs 28,8 ± 1,4 mg/dl; p <0,01). Furthermore, serum LDL cholesterol levels tended to be increased by SRL treatment as compared to the vehicle treated group (16,2 ± 1,3 vs 12,9 ± 1,0 mg/dl; p =0,0575) (Figure 22C).

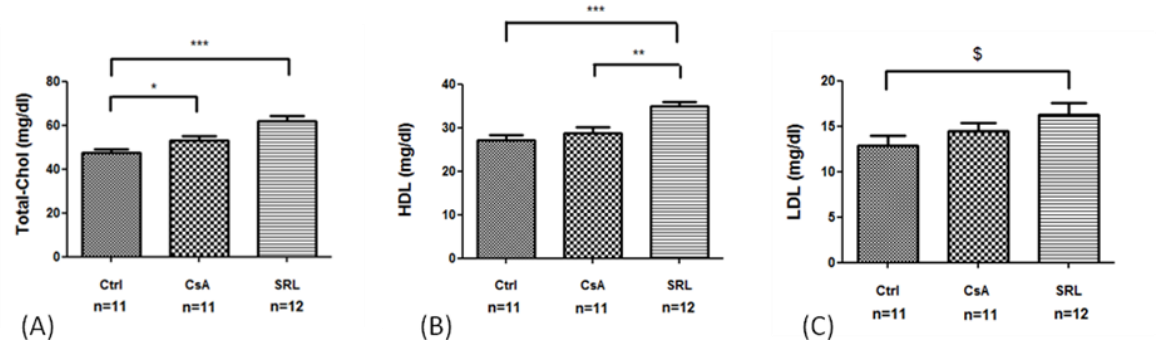

Figure 22 - Serum fed cholesterol levels in Wistar rats after 6 weeks of cyclosporin A and sirolimus treatments. (A) Total cholesterol (Total-Chol); (B) High-density lipoprotein (HDL); (C) Low-density lipoprotein (LDL). Cholesterol concentrations were measured as reported in Matherials & Methods. The results are representative of two independent experiments. Data are shown as mean ±S.E.M. Ctrl, control; CsA, cyclosporin A; SRL, sirolimus. t-test, **p* <0.05, ***p* <0.01 or ****p* <0.001; $=0,0575.

4.2. Triglyceride levels in serum, liver and muscle

Figure 23 shows that both cyclosporin A and sirolimus treatments lead to significantly elevated trygliceride levels in blood (143,3 ± 24,6 vs 78,8 ± 11,7 mg/dl, p<0,05 and 155,5 ± 14,1 vs 78,8 ± 11,7 mg/dl, respectively, p<0,01), compared to vehicle (Figure 23A). TGs were also assessed in liver and muscle in the three groups. These results show significantly higher TGs levels in both tissues (3,0 ± 0,3 vs 1,8 ± 0,1 mg/dl/mg tissue and 2,5 ± 0,5 vs 1,0 ± 0,2 mg/dl/mg tissue, respectively; p=0,05), in the sirolimus group, compared to vehicle, resulting in a process of steatosis caused by this drug. A rising trend was observed in the CsA treated group (Figure 23B).

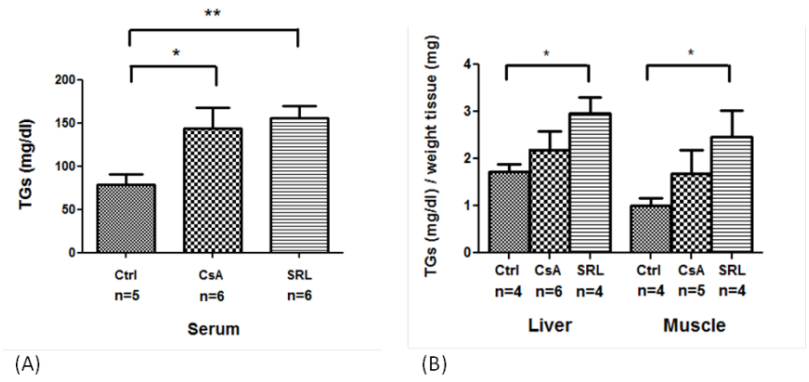

Figure 23 - Fed trygliceride levels in Wistar rats after 6 weeks of cyclosporin A and sirolimus treatments in serum, liver and muscle. (A) Triglycerides (TGs) in serum; (B) Triglycerides in liver and muscle (values are normalized by the weight of the tissue used for the measurement). TGs were measured by different methods as reported in Matherials & Methods. Data are shown as mean ± S.E.M. Ctrl, control; CsA, cyclosporin A; SRL, sirolimus. t-test, **p* <0.05 or ***p* <0.01.

5. Glucose uptake

5. 1 Adipocyte isolation, diameter and weight

Weight and diameter of adipocytes were obtained after collagenase digestion of epididymal fat pads, as described earlier, for either vehicle, CsA or SRL treated animals. Differences in either cell weight or cell size between the three treated groups were not statistically significant. However, we observed that adipocytes of CsA and SRL treated rats tended to be heavier than those of vehicle treated rats (Figure 24).

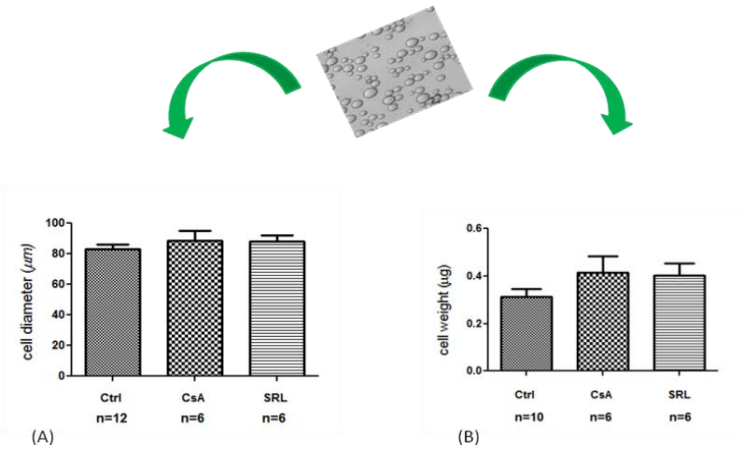

Figure 24 - Adipocyte diameter and weight from epididymal adipose tissue, of 16 week old Wistar rats, after 6 weeks of cyclosporin A and sirolimus treatments. (A) The diameter of 100 consecutive cells from each rat was measured after adipocyte isolation. Cell size is expressed in µm. (B) Cell weight was obtained after assessing the triglyceride mass in 500 µl cell suspension, as described in Materials & Methods. Cell weight is expressed in µg. The results are representative of two experiments. Ctrl, control; CsA, cyclosporin A; SRL, sirolimus. Data are shown as mean ±S.E.M.

5.2 Glucose uptake assay

As illustrated on Figure 25, incubation of isolated adipocytes from digested epididymal adipose tissue of 16 weeks old vehicle treated rats with a maximal insulin concentration (10 nM) resulted in a 3,8 fold increase in the insulin-stimulated glucose uptake over basal. However, when glucose uptake was measured in either the CsA and SRL treated groups we observed a significant decrease of the insulin-stimulated glucose uptake of 51,3 and 50,5% respectively, compared to the vehicle treated group, demonstrating an impaired insulin-stimulated rate of glucose transport. Moreover, with CsA treatment, we observed an increase in the basal (without insulin incubation) glucose uptake compared to that of the vehicle treated group (122,1 ± 15,8 vs 64,3 ± 12,06

fl/cell/sec; p<0,05). On the other hand, with SRL we observed a decrease in the basal glucose uptake, compared to vehicle (27,3 ± 3,4 vs 64,3 ± 12,06 fl/cell/sec; p<0,05).

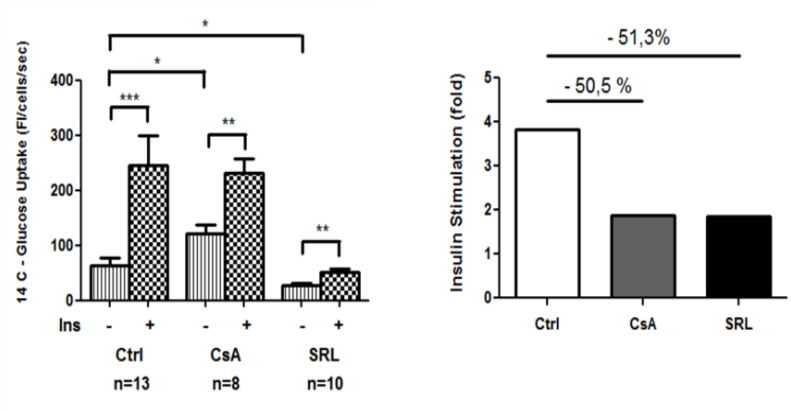

Figure 25 - Glucose transport in isolated rat adipocytes performed after 6 weeks of cyclosporin A and sirolimus treatments. Adipocytes were incubated with or without insulin (10 nM) for 10 min (left). [U-^{14}C] glucose was then added and the incubations were continued for a further 30 min. Cells were prepared as described in Materials & Methods. Experiments were performed in triplicate and expressed as cellular glucose clearance (femtoliter (fl) per cell per second). The results are also shown as the difference in insulin stimulation (fold) relative to basal of glucose uptake between Ctrl, CsA and SRL groups (right). The results are representative of two experiments. Data are shown as mean ±S.E.M. Ctrl, control; CsA, cyclosporin A; SRL, sirolimus. t-test, **p* <0.05, ***p* <0.01 or ****p* <0.001.

6. Gene and protein expression

6.1. IRS-1, Glut4 and Glut1 expression in liver, muscle and fat

To understand the effect of cyclosporin A and sirolimus on the glucose uptake after insulin stimulation, gene expression for IRS-1 and Glut4 was assessed. We observed that cyclosporin A treatment caused an inhibition of IRS-1 gene expression in liver (0,026 ± 0,003 vs 0,036 ± 0,003; p<0,05) (Figure 26A) and in perirenal fat (0,015 ± 0,003 vs 0,073 ± 0,013; p<0,05), compared to vehicle (Figure 26C), while in muscle, IRS-1 gene expression was not changed (Figure 26B). Similarly, the sirolimus treatment caused a significant decrease in IRS-1 gene expression in both muscle (0,006 ± 0,0009 vs 0,013 ± 0,002; p =0,0556) (Figure 26B) and perirenal fat (0,02 ± 0,006 vs 0,07 ± 0,013; p <0,05) (Figure 26C), but no differences were found in the liver (Figure 26A). Moreover, Glut4 gene expression was also assessed in muscle and perirenal fat. While sirolimus treatment caused a decrease in Glut4 in muscle (0,009 ± 0,002 vs 0,016 ± 0,002; p <0,05) (Figure 26D), no significant differences in Glut4 gene expression were observed in perirenal fat (Figure 26E). On the other hand, with CsA treatments we observed no significant

differences in the Glut4 gene expression in either tissue, even though, the tendency for a decrease in Glut4 expression was observed (Figure 26D), particularly in perirenal fat (0,056 ± 0,012 vs 0,094 ± 0,014; p=0,0866) (Figure 26E).

Furthermore, we proposed to determine if the impaired basal glucose uptake after sirolimus treatment could be explained by changes in Glut1 gene expression. A significant decrease in Glut1 expression was found with the sirolimus treatment in both liver (0,001 ± 0,0003 vs 0,0029 ± 0,0003; p<0,001) (Figure 26F) and in perirenal fat (0,0005 ± 0,0002 vs 0,0023 ± 0,0003; p <0,01) (Figure 26H), while in muscle there was only a tendency for a decrease (p=0,0866) (Figure 26G). Moreover, a significant decrease in Glut1 gene expression was also found in liver (0,0016 ± 0,0003 vs 0,0029 ± 0,0003; p <0,05) (Figure 26F) with CsA treatment compared to vehicle.

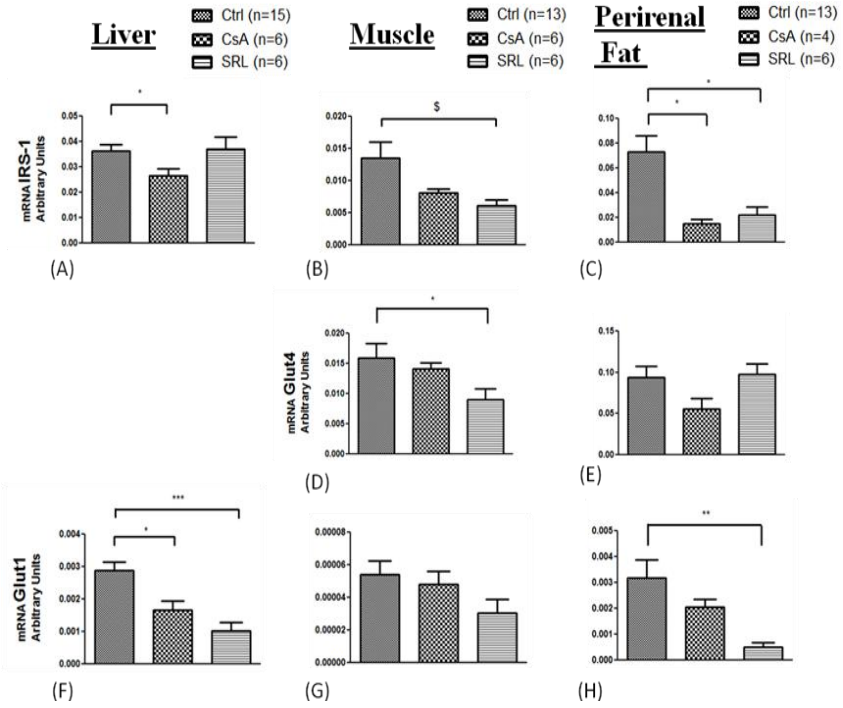

Figure 26 – IRS-1, Glut4 and Glut1 gene expression, after 6 weeks of cyclosporin A and sirolimus treatments. Total RNA was isolated from liver, muscle and perirenal adipose tissue, as described in Materials & Methods. The mRNA levels were assessed by quantitative real-time RT-PCR for IRS-1 in liver (A), muscle (B) and perirenal fat (C); for Glut4 in muscle (D), and perirenal fat (E); for Glut1 in liver (F), muscle (G) and perirenal fat (H). Gene expression was normalized using the reference gene GAPDH. Experiments were performed in duplicate. Results are representative of two representative experiments. Data are shown as mean ±S.E.M. Ctrl, control; CsA, cyclosporin A; SRL, sirolimus. t-test, **p* <0.05, ***p* <0.01 or ****p* <0.001; $ *p*=0,0556.

6.2. IL-6, TNFα and Adiponectin gene expression in fat

IL-6, TNFα and Adiponectin gene expression were measured on RNA extracted from perirenal fat in order to investigate how lipolysis and insulin sensitivity might be

modulated after 6 weeks of either CsA or SRL treatments through these adipokines/inflammatory mediators.

Sirolimus treatment correlated with an increase in IL-6 gene expression (0,0004 ± 7,739e-005 vs 6,255e-005 ± 7,157e-006; p <0,05), compared to vehicle (Figure 27A), while it had no effects on the gene expression levels of either TNFα (Figure 27B), or adiponectin. Adiponectin expression was significantly decreased only for CsA treatments (1,954 ± 0,138 vs 3,061 ± 0,672; p<0,05), compared to vehicle (Figure 27C).

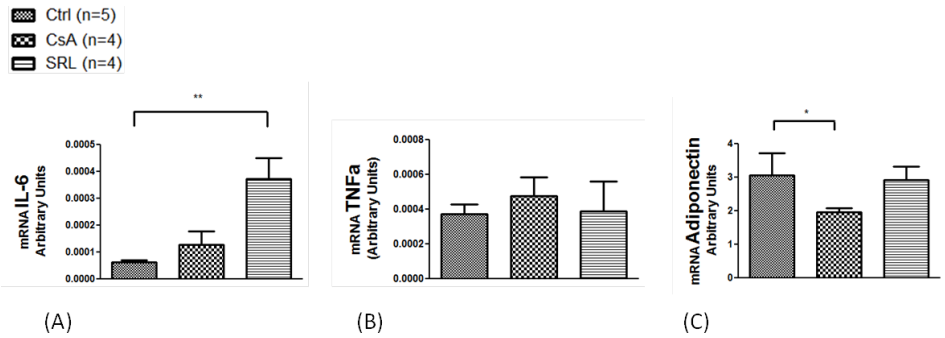

Figure 27 – IL-6, TNFα and adiponectin gene expression after 6 weeks of cyclosporin A and sirolimus treatments. Total RNA was isolated from perirenal adipose tissue, as described in Materials & Methods. mRNA levels were assessed by quantitative real-time RT-PCR for IL-6 (A), TNFα (B) and Adiponectin (C). Gene expression was normalized using the reference gene GAPDH. Experiments were performed in duplicate Results are representative of two representative experiments. Data are shown as mean ±S.E.M. Ctrl, control; CsA, cyclosporin A; SRL, sirolimus. t-test, *p <0.05, **p <0.01 or ***p <0.001.

6.3. Expression of genes and proteins involved in gluconeogenesis and lipogenesis

6.3.1. DGAT1 and PGC1α expression in liver, muscle and fat

As showed on Figure 28, in perirenal fat, cyclosporin A and sirolimus treatments significantly reduced the gene expression levels of DGAT1 (0,089 ± 0,009 vs 0,258 ± 0,033, p<0,05 and 0,094 ± 0,010 vs 0,258 ± 0,033, p<0,01, respectively), compared to vehicle (Figure 28C). A tendency for a decrease was also found in muscle (Figure 28B) compared to vehicle and no significant effects were found in liver (Figure 28A). Moreover, our results show that both cyclosporin A and sirolimus treatments resulted in a significant decrease in PGC1α gene expression in liver (0,0013 ± 0,0002 and 0,0015 ± 0,0002 vs 0,0038 ± 0,0007, respectively; p<0,05), muscle (0,0012 ± 0,0001 and 0,0007 ± 0,0001 vs 0,0022 ± 0,0004, respectively; p<0,05) and perirenal fat (0,0003 ± 8,114e-005 and 0,0002

± 3,316e-005 vs 0,0023 ± 0,0006, respectively; p<0,05), compared to vehicle (Figure 28D, E, F).

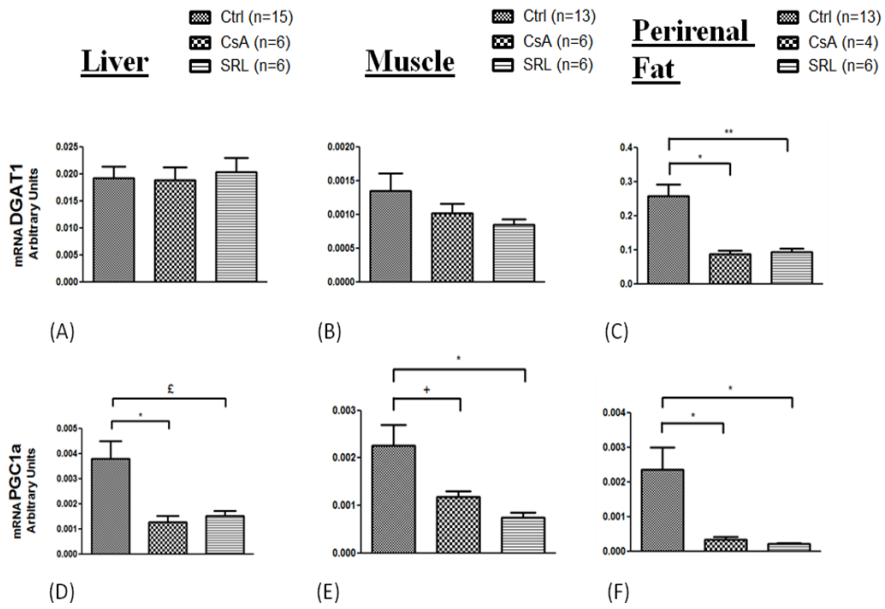

Figure 28 - DGAT1 and PGC1α gene expression, after 6 weeks of cyclosporin A and sirolimus treatments. Total RNA was isolated from liver, muscle and perirenal adipose tissue, as described in Materials & Methods. mRNA levels were assessed by quantitative real-time RT-PCR in liver (A, D), muscle (B, E) and perirenal fat (C, F). Gene expression was normalized using the reference gene GAPDH. Experiments were performed in duplicate. The results are representative of two independent experiments. Data are shown as mean ±S.E.M. Ctrl, control; CsA, cyclosporin A; SRL, sirolimus. t-test, **p* <0.05 or ***p* <0.01; £ *p*=0,0522, + *p*=0,0511.

6.3.2. FoxO1: gene and protein expression

FoxO1 gene expression, as well as protein amount were measured in order to understand its metabolic function, such as gluconeogenesis, after 6 weeks of either cyclosporin A and sirolimus treatment. As Figure 29 shows, we observed, in liver, an increase in FoxO1 gene expression caused by both CsA (0,246 ± 0,0204 vs 0,136 ± 0,013; p<0,001) and SRL (0,266 ± 0,04357 vs 0,136 ± 0,013; p<0,01) treatments compared to vehicle (Figure 29A). Protein amount showed the same trend in liver, for both CsA (2,320 ± 0,143 vs 1,734 ± 0,108; p<0,05) and SRL (2,408 ± 0,229 vs 1,734 ± 0,108; p=0,0564) (Figure 29D) treatments compared to vehicle. No further changes were observed in muscle, either in terms of gene (Figure 29B) or protein expression (Figure 29E) compared to vehicle. Conversely, in perirenal fat, treatments resulted in a down-regulation in FoxO1 gene expression (0,0521 ± 0,0064 for CsA and 0,0643 ± 0,0104 for SRL vs 0,1392 ± 0,0169; p<0,05) compared to vehicle (Figure 29C), but no effect was observed in the protein expression (Figure 29F).

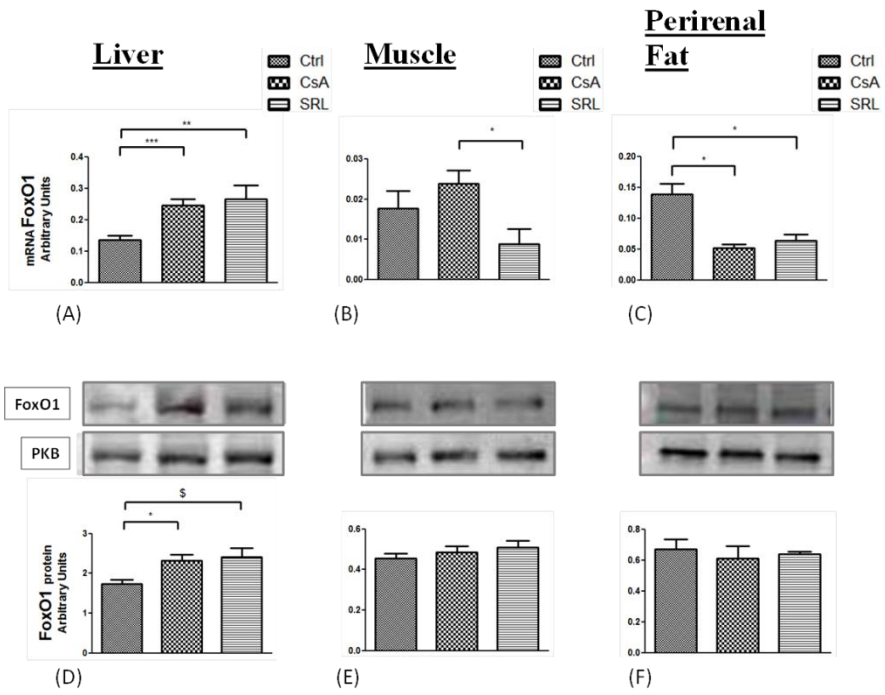

Figure 29 - FoxO1 gene and protein expression, after 6 weeks of cyclosporin A and sirolimus treatments. Total RNA was isolated from liver, muscle and perirenal adipose tissue, as described in Materials & Methods. mRNA levels were assessed by quantitative real-time RT-PCR (Ctrl, n=15; CsA, n=6; SRL, n=6) in liver (A), in muscle (B) and in perirenal fat (C). Gene expression was normalized using the reference gene GAPDH. Cell lysates were obtained from liver (D), muscle (E) and perirenal fat (F). Subsequent immunoblotting analysis was performed. Equal protein amount was subjected to SDS-PAGE, transferred to PVDF membranes and subjected to WB analysis as described before, using FoxO1 antibody, normalized to Akt/PKB antibody. The blot shown is representative of at least 3 independent experiments yielding similar results (n=3/group). Data are shown as mean ±S.E.M. Ctrl, control; CsA, cyclosporin A; SRL, sirolimus. t-test, **p* <0.05, ***p* <0.01; $, *p*=0,0564

6.3.3. SREBP-1: gene and protein expression

SREBP-1 gene and protein expressions were also assessed. In liver, we observed a significant increase in SREBP-1 gene expression in both CsA (0,7097 ± 0,1082 vs 0,1950 ± 0,0363; p<0,001) and SRL (0,4898 ± 0,0529 vs 0,1950 ± 0,0363; p<0,001) treated groups compared to vehicle (Figure 30A), while its protein expression seemed to be down regulated for the SRL treatment (6,606 ± 0,6434 vs 10,71 ± 0,7625; p<0,05) compared to vehicle (Figure 30D). Moreover, in contrary to SREBP-1 mRNA concentration, that was clearly increased in muscle for the CsA treatment (0,0695 ± 0,0151 vs 0,0294 ± 0,0049; p<0,01) (Figure 30B), the SREBP-1 protein expression is reduced in muscle of CsA treated rat (5,654 ± 0,267 vs 7,635 ± 0,428; p<0,05) (Figure 30E) as compared to the vehicle treated group. No differences were observed in perirenal fat for either gene (Figure 30C) or protein expression (Figure 30F).

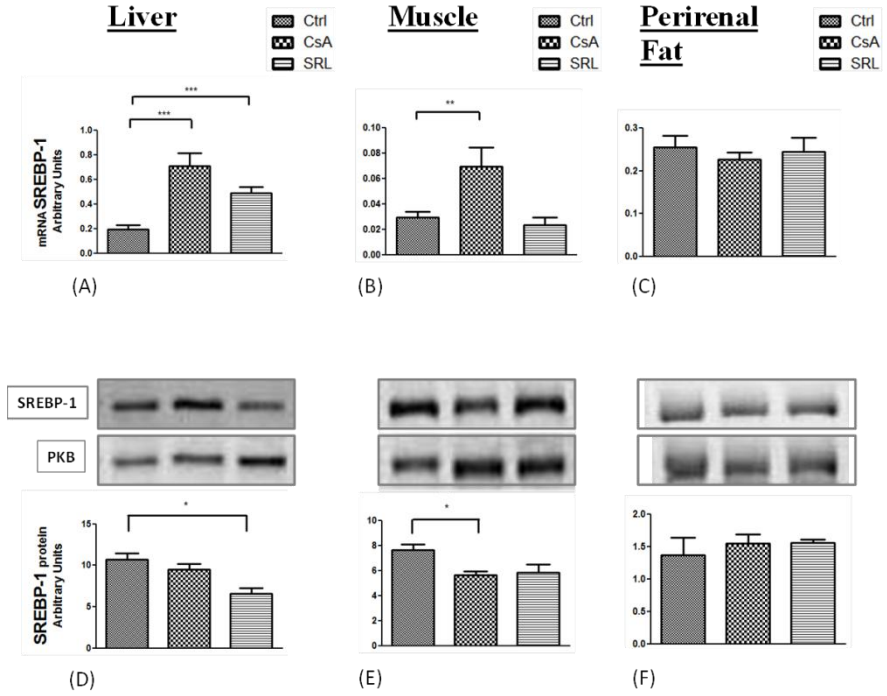

Figure 30 - SREBP-1 gene and protein expression after 6 weeks of cyclosporin A and sirolimus treatments. Total RNA was isolated from liver, muscle and perirenal adipose tissue, as described in Materials & Methods. mRNA levels were assessed by quantitative real-time RT-PCR (Ctrl, n=15; CsA, n=6; SRL, n=6) in liver (A), in muscle (B) and in perirenal fat (C). Gene expression was normalized using the reference gene GAPDH. Cell lysates were obtained from liver (D), muscle (E) and perirenal fat (F). Subsequent immunoblotting analysis was performed. Equal protein amount was subjected to SDS-PAGE, transferred to PVDF membranes and subjected to WB analysis, using the antibody against SREBP-1, normalized to the Akt/PKB antibody. The blot shown is representative of at least 3 independent experiments yielding similar results (n=3/group). Data are shown as mean ±S.E.M. Ctrl, control; CsA, cyclosporin A; SRL, sirolimus. t-test, **p* <0.05, ***p* <0.01 or ****p* <0.001.

6.3.4. ChREBP: gene and protein expression

As showed on Figure 31, sirolimus treatment caused an up-regulation in ChREBP gene expression in liver (1,071 ± 0,1848 vs 0,6754 ± 0,0504; p<0,05) (Figure 31A), even if the increase in the protein amount is not significant, compared to vehicle (Figure 31D). Conversely, in muscle, either drug treatment, resulted in a tendency for a decrease in either ChREBP gene or protein expression compared to vehicle. Finally, in perirenal fat, ChREBP gene expression but not protein expression (Figure 31F), was down regulated by SRL treatment (0,2369 ± 0,0703 vs 0,5377 ± 0,0734; p<0,05), while only a trend for a decrease was observed for CsA. (Figure 31C).

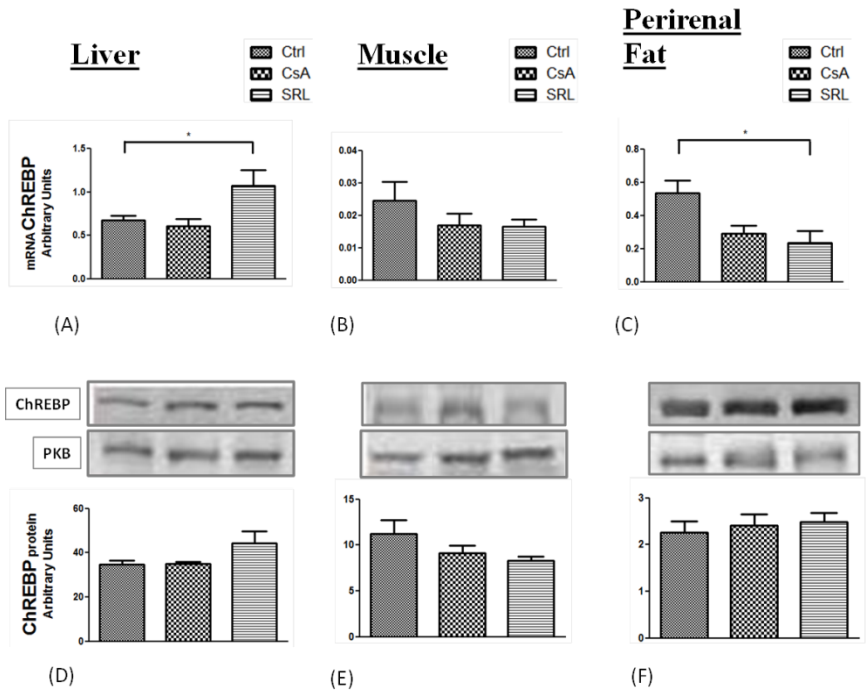

Figure 31 - ChREBP gene and protein expression after 6 weeks of cyclosporin A and sirolimus treatments. Total RNA was isolated from liver, muscle and perirenal adipose tissue, as described in Materials & Methods. mRNA levels were assessed by quantitative real-time RT-PCR (Ctrl, n=15; CsA, n=6; SRL, n=6) in liver (A), in muscle (B) and in perirenal fat (C). Cell lysates were obtained from liver (D), muscle (E) and perirenal fat (F). Subsequent immunoblotting analysis was performed. Equal protein amount was subjected to SDS-PAGE, transferred to PVDF membranes and subjected to WB analysis, using the ChREBP antibody, bands were normalized to the Akt/PKB antibody. The blot shown is representative of at least 3 independent experiments yielding similar results (n=3/group). Data are shown as mean ±S.E.M. Ctrl, control; CsA, cyclosporin A; SRL, sirolimus. t-test, **p* <0.05.

6.3.5. ACC1: gene and protein expression

Figure 32 shows a significant increase in ACC1 gene expression in muscle caused by CsA (0,0012 ± 0,0003 vs 0,0004 ± 6,173e-005; p<0,01) compared to vehicle (Figure 32B). However, the protein amount did not show any differences between the groups. Conversely, in fat we observed a slight decrease in ACC1 expression caused by both CsA (0,118 ± 0,028 vs 0,288 ± 0,048; p=0,0603) and SRL (0,124 ± 0,049 vs 0,288 ± 0,048; p=0,0777) treatments compared to vehicle (Figure 32C). However, protein amount was not measured due to lack of tissue. No further changes were observed in gene expression in liver (Figure 32A), in spite of an increase in protein amount (Figure 32D) for CsA treatment.

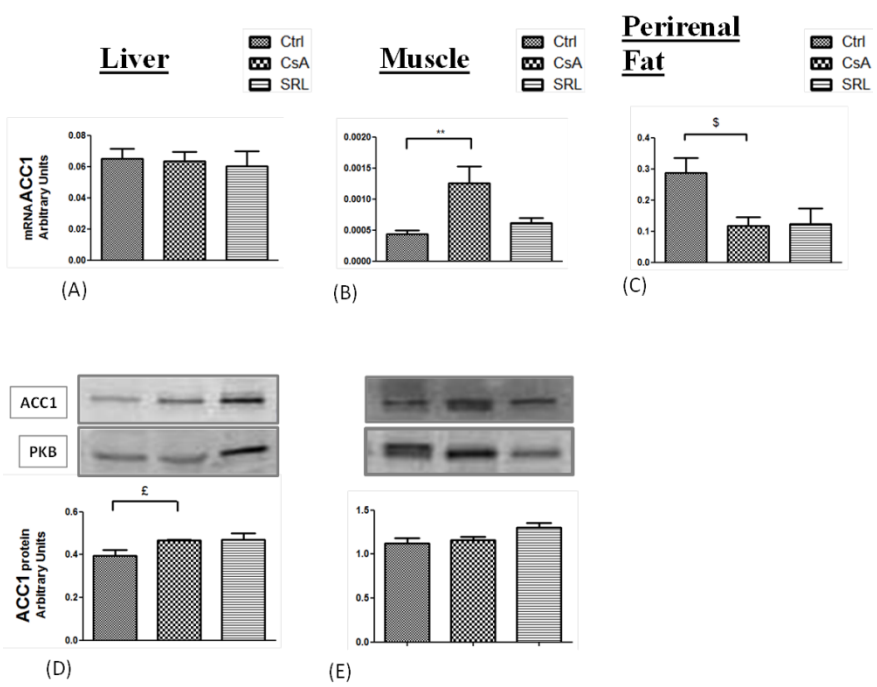

Figure 32 - ACC1 gene and protein expression after 6 weeks of cyclosporin A and sirolimus treatments. Total RNA was isolated from liver, muscle and perirenal adipose tissue, as described in Materials & Methods. mRNA levels were assessed by quantitative real-time RT-PCR (Ctrl, n=15; CsA, n=6; SRL, n=6) in liver (A), in muscle (B) and in perirenal fat (C). Cell lysates were obtained from liver (D) and muscle (E). Subsequent immunoblotting analysis was performed. Equal protein amount was subjected to SDS-PAGE, transferred to PVDF membranes and subjected to WB analysis, using the ACC1 antibody, bands were normalized to the Akt/PKB antibody. The blot shown is representative of at least 3 independent experiments yielding similar results (n=3/group). Data are shown as mean ±S.E.M. Ctrl, control; CsA, cyclosporin A; SRL, sirolimus. t-test, ***p* <0.01; .$ *p*=0,0603; £, *p*=0,0556.

Chapter IV. Discussion

IV - Discussion

In this thesis I analyzed the molecular mechanisms involved in the development of well-recognized complications associated with immunosuppressive therapies, such as dyslipidemia and NODAT, focusing on long term effects of cyclosporin A and sirolimus *in vivo* treatment on glucose and lipid metabolism. The animals in our model were healthy and received no further intervention except for treatment with CsA, SRL or vehicle.

In the present study, we show that the mTOR inhibitor sirolimus produced a decrease in the weight gain during the last 2 weeks of treatment compared to either vehicle or CsA treatment. In other words SRL treatment attenuated the physiologically normal gain of body weight. Rovira J et al. [206] observed a similar trend in 15 week old Wistar rats after 12 weeks of SRL treatment (1.0 mg/kg) compared to control and the same was observed in kidney transplant patients two years after transplantation, compared to those with CsA treatment. Further studies have shown failure to weight gain with rapamycin in other rodent models [174, 207, 208]. A possible explanation may be the effects of SRL on metabolic regulation and cell growth as mTOR is a nutrient sensor and a crucial key regulator for special cellular growth and metabolic markers, such as, p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4Ebinding protein 1 (4E-BP1) [209]. Both are implicated in protein synthesis. Furthermore, mTOR is a regulator of transcription by either inhibiting or activating cellular processes, including autophagy, regulation of ribosome biogenesis and metabolism [210, 211]. On the other hand, we cannot exclude an anorexigenic effect of this drug, as observed in other rat models [208, 212], as the amount of food and water intake were not evaluated.

Furthermore, we could show that SRL treated rats had higher fed glucose levels in spite of showing the same fed insulin concentration, thus suggesting that SRL treatment in our animals impaired insulin sensitivity. In addition, other *in vivo* studies have shown that chronic treatments with SRL (2 mg kg^{-1} day^{-1}) markedly affected glucose and insulin homeostasis as reflected by hyperglycemia and hyperinsulinemia observed in these models [208, 212]. On the other hand, glucose levels, after an overnight fast, were unchanged as shown before a glucose tolerance test. Fasted insulin concentrations have not been

measured. Nevertheless, other *in vivo* SRL treatment studies have shown that fasted insulin levels were unaltered [206] or increased [207, 212, 213], perhaps suggesting a decrease in insulin sensitivity. Furthermore, we didn't observe any differences in either the fed or fasted glucose, as well as in the fed insulin levels, with the CsA treatment.

However, during a GTT, CsA treated rats presented higher glucose levels 15 minutes after a glucose bolus, followed by a rapid return to normal in their glycemia. Insulin concentrations were not measured during this test, but similar studies have been observed, in either *in vivo* animal models or in human studies. Cha et al. performed an ipGTT in male Wistar rats, after 7 days of treatment, with 50 mg kg^{-1} day^{-1}·CsA, showing markedly impaired glucose tolerance with significantly decreased basal and glucose-stimulated serum insulin levels [214]. While Menegazzo et al. observed, in a rat model, a decrease in insulin secretion after 4 week of SRL treatment (10 mg kg^{-1} day^{-1}), but no changes were evaluated after shorter treatment times [215]. Moreover, David-Neto E et al. performed an oral GTT at days 30, 60, 180, and 360 in patients after renal transplantation, showing that the incidence of impaired glucose tolerance or diabetes mellitus reached a peak at 60 days and decreased at 1 year, while the insulin secretion decreased at day 60, showing a condition of IGT or DM development [216]. Although the majority of the studies that have examined the diabetogenic effects of CsA indicate that this drug inhibits insulin production and secretion from the beta-cells of the islets of Langerhans [217-220], a reduction in peripheral insulin sensitivity has also been suggested [183, 221, 222]. Interestingly, a recent study performed in healthy human volunteers, treated with clinically relevant doses of cyclosporin A, showed increase in insulin sensitivity without affecting insulin secretion [223]. Additionally, we observed glucose intolerance after SRL treatment as demonstrated by the impaired glucose excursion during the glucose tolerance test. We believe that this SRL effect is due to insulin resistance, in part through the induction of hepatic gluconeogenesis by enhancement of the expression of gluconeogenic enzymes and nuclear recruitment of important gluconeogenic transcriptional regulators as some studies have pointed out [212, 213], in agreement with our results. Some further studies have also shown that SRL may cause impairment of glucose tolerance associate to enhanced glucose-induced insulin, during a GTT [208, 212]. These observations allow us to suggest that our animals, after 6 weeks of SRL treatment, show IGT. Moreover, contradictory results have been reported on the presence or the absence of beneficial effects of SRL on islet mass and

function [224, 225], while others described an impairment of beta-cell survival, insulin secretion and islets engraftment [226, 227]. Interestingly, adipose-specific knockout mice for rictor (mTORC2) have an enlarged pancreas and are hyperinsulinemic, suggesting a potential for cross-talk between mTOR signalling in adipose tissue and pancreatic function [228].

Furtheremore, the lipid profile was also evaluated in each group, showing that SRL is more involved in causing hyperlipidemia than cyclosporin A is. Indeed, our study shows a significant increase in trygliceride levels in sirolimus treated rats, in both serum and tissues, resulting in a process of tissue steatosis caused by the drug. However, other studies have observed that SRL treatment could prevent [208] or causes no changes in fat accumulation and hepatic steatosis [212]. This maybe be due to different treatment and experimental conditions. In addition, previous experimental and clinical studies [123, 174, 212, 229, 230] have observed an impaired lipid profile in circulation after CsA/SRL treatments. According to these data, our treated animals presented higher levels of both serum tryglicerides and cholesterol levels in circulation, especially in the sirolimus treated group, compared to vehicle. Hyperlipidaemia, potentially resulting from increased adipose tissue lipolysis or hepatic TG synthesis, has been suggested to represent one of the factors contributing to peripheral insulin resistance following systemic mTOR inhibition as indicated by several studies [123, 212, 231, 232]. In addition, in the CsA treated rats, we also observed higher levels of total cholesterol in circulation, as well as increased TGs levels compared to control as already reported by Wu et al. [233]. The exact mechanism underlying cyclosporin-induced hyperlipidemia has not been elucitaded completely, but these data are in agreement with increased lipogenic gene expression in liver observed in this work. Studies in nontransplant subjects who received CsA show increases in plasma cholesterol with elevation in plasma LDL levels [234], probably because of an inhibition by CsA of steroid 26 hydroxylase, an important enzyme that enables bile acid synthesis from cholesterol [131]. However in some patients hyperlipidemia occurs secondary to an underlying genetic predisposition and/or environmental factors [235]

One of the possible causes for the differences in body weight could be due to a reduction in adipose tissue mass, however during this experiment tissues were not weighed. Surprisingly, in our study both adipocyte cell weight and size were measured but

we did not find any statistical differences in our results with the number of animals studied, as opposed to several other studies [206, 207, 212, 178], were they observe inhibition in cell growth and the increase in catabolic processes by SRL. In addition, *in vitro* studies shows that SRL treatment causes inhibition of the clonal expansion and differentiation of the pre adipocytes 3T3-L1 and induces differentiation of mature adipocytes (adipogenesis) [236]. We did however, find that adipocytes of CsA and SRL treated rats tended to be heavier than those of vehicle treated rats, suggesting that CsA and SRL treated cells may accumulate more lipid then the vehicle treated cells.

Moreover, our results show that both CsA and SRL treated groups showed a significant decrease in the insulin-stimulated glucose uptake, in isolated rat adipocytes compared to the vehicle treated group. These results may in part explain the impaired glucose tolerance observed during a GTT. Also, similar results show that CsA has a concentration-dependent inhibitory effect on basal and insulin-stimulated 14C-glucose uptake in human isolated adipocytes [221]. Furthermore, recent studies in both mice and humans have indicated that treatment with calcineurin inhibitors is associated with reduced insulin sensitivity in peripheral tissues, as well as, impaired endothelial function [214, 237-239]. Additionally, consistent with the study of Pereira et al. [201] we showed that SRL suppresses both basal and insulin stimulated glucose uptake, compared to vehicle. However the effects of SRL treatment on the insulin response are still debated. Rovira et al. [206] show that SRL-treated rats had lower glycemia in spite of showing the same insulin concentration, thus suggesting that SRL treatment in their animals enhances insulin sensitivity. Um et al. [240] observed a similar effect in their mice. The S6K1 knock-out mice they studied showed higher insulin sensitivity. They attributed this effect to the absence of the physiological negative feedback mechanism that S6K1 exerts on IRS proteins. In addition, previous conflicting, *in vitro*, studies were found. While Cho et al. [178] have suggested that long-term treatment of 3T3-L1 adipocytes with sirolimus reduces their insulin dependent glucose uptake capacity, others [241] have reported that short-term sirolimus treatment (1 h) of 3T3-L1 cells and differentiated human pre-adipocytes in states of increased activity of mTOR/S6K pathway, relieve the repression of IRS-1/PI3–K/PKB signalling leading to increased insulin stimulated glucose transport. These findings suggest that sirolimus may have different effects on glucose uptake dependent on the elevated basal mTOR/S6K activation in adipocytes.

Furthermore, we proposed to assess whether an altered expression of Glut transporters could contribute to the inhibition of glucose uptake. We suggest that an impaired translocation of Glut4 might be a likely cause of the attenuated insulin signaling-stimulated glucose uptake observed following either CsA or SRL treatments. We have however not been able to perform these studies due to lack of tissue. However, Pereira et al. have shown that therapeutic concentrations of CsA can inhibit glucose uptake, in human subcutaneous and omental adipocytes and in L6 muscle cells, by removing Glut4 from the cell surface via increased endocytosis rates, independently of the insulin signalling action. [242]. However, we measured Glut4 gene expression but no significant change was observed either for CsA or SRL treatments in perirenal fat, but so far we did not measured Glut4 protein expression in either group, therefore, we cannot exclude a possible decrease in protein amount, or more importantly, the insulin-induced translocations of the protein from the vesicles to the plasma membrane with the respective treatment as some studies have shown [208, 232]. The molecular mechanism responsible for the decrease in insulin sensitivity observed in adipocytes under these treatments, may be attributed to a down-regulation in IRS-1 gene expression found in perirenal fat for both treatments, compared to vehicle, however, due to the lack of tissue we have not been able to measure IRS-1 protein expression. Moreover, a down-regulation of IRS-1 and Glut4 gene expression, after SRL treatment and a decreased level of IRS-1 gene expression in liver for CsA treated rats may be responsible for insulin resistance at the level of the muscle and liver. Consistent with our data Deblon et al. found that chronic SRL administration also down-regulated muscle Glut4 and Glut1 gene and protein expression in rat skeletal muscle as well as IRS-1 protein in L6 myotubes [208]. *In vitro* analyses have previously shown that expression of glucose transporters may also be modulated by mTOR inhibition [243, 193]. Glucose uptake in skeletal muscle is mediated through insulin-dependent and independent mechanisms, all requiring appropriate expression of specific glucose transporters. More specifically, Glut1 mediates basal glucose transport, whereas Glut4 is responsible for insulin-stimulated glucose uptake [244]. For that reason, we have proposed a correlation between impaired basal glucose uptake by SRL treatment and the decrease in Glut1 gene expression observed in both perirenal fat and liver, as also confirmed by others [193, 245]. We still need though, to measure Glut1 protein expression. On the other hand, CsA treatment enhances

the basal glucose uptake compared to that of the vehicle treated group, although the mechanism by which it happens remains unclear.

Importantly,Bastard JP et al. have shown [246], that cytokines and adipokines, produced by adipocytes, may play a major role in glucose and lipid metabolism. In particular, increased levels of IL-6 and TNFα seem to induce lipolysis via activation of PKA and MAPKs p44/42, respectively [247], contributing to insulin resistance and dyslipidemia. Adiponectin, on the other hand, inhibits lipolysis through a 5'-AMP-activated protein kinase (AMPK) dependent mechanism working as an insulin sensitizing adipokine [248]. We demonstrate that SRL can enhance IL-6 adipose tissue gene expression *in vivo*, indicating that SRL may contribute to increased levels of IL-6 in circulation.IL-6 may act as an autocrine and/or paracrine mediator, stimulating lipolysis, although the protein amount was not measured. In accordance, Pereira et al a have demonstrated that SRL, as well as CsA in an *in vitro* treatment can enhance IL-6 gene expression in adipose tissue [249]. In addition, the same study shows an increased IL-6 secretion following *in vitro* SRL treatment of adipocytes. No changes were observed in TNFα gene expression. Indeed TNFα does not seem to be sensitive to sirolimus according to several other studies [250, 251]. Nevertheless we cannot exclude an increase in protein synthesis and its increasing circulating levels. Furthermore, CsA treatment also suppressed adiponectin gene expression, suggesting a higher resistance to insulin caused by this drug.

Expression of genes involved in gluconeogenesis/lipogenesis and lipid storage were also assessed, in order to understand which pathways might be involved in the impaired lipid profile observed in our rat model.

DGAT1, one of two known DGAT enzymes that catalyze the final step in triglyceride synthesis from diacylglycerol and acyl-CoA, was observed to have a significant reduction in gene expression only in perirenal fat, after both drug treatments [28]. Findings from genetically modified mice, as well as pharmacological studies suggest that inhibition of DGAT1 is a promising strategy for the treatment of obesity and type 2 diabetes [28]. However our results do not allow us to take into account DGAT as a potential cause of hypertriglyceridemia, in liver and muscle, as DGAT expression is not different from vehicle, but DGAT decrease in fat may suggest an reduced lipogenesis in

this tissue by these drug, as previously demonstrated [249, 231, 252] and in accordance with our further results. We have not measured the protein amount in our study and therefore we can't make a solid conclusion regarding the results. Interestingly, DGAT1-/- mice are healthy and fertile and have no changes in triglyceride levels [253].

Furthermore, PGC1α gene expression was quantified as it plays an important role in insulin sensitivity and T2D, being essential in mitochondria biogenesis and glucose/fatty acid metabolism [254]. Fasting produces a robust increase of PGC-1α expression, which in turn, stimulates hepatic gluconeogenesis and fatty acid oxidative metabolism [25, 255]. Moreover PGC-1α stimulates mitochondrial biogenesis and promotes the remodeling of muscle tissue to a fiber-type composition that is metabolically more oxidative and less glycolytic in nature, participating in the regulation of both carbohydrate and lipid metabolism [254]. In addition, it has been observed that expression of PGC-1α is downregulated in muscle of T2D subjects [76, 256], making it an inviting target for pharmacological intervention in the treatment of obesity and T2D [254]. These findings support our results. PGC1α gene expression was greatly reduced in liver, muscle and adipose tissues, with either drug treatment, indicating the diabetologic effect of these drugs, by decreasing the transcription of metabolic and mitochondrial genes, resulting, in turn, in the inhibition of lipid oxidation and increasing tissue steatosis. Forthermore, as observed by Puigserver et al., PGC-1α binds and co-activates FoxO1 interacting in the execution of the insulin-regulated gluconeogenesis [18]. In turn, FoxO1 binds to the promoter regions of those genes encoding key gluconeogenic enzymes such as PEPCK and G-6-Pase.

Moreover, enhanced gene and protein expression of FoxO1 were observed in liver for both CsA and SRL, suggesting an increase in gluconeogenesis, as previously observed by Houde et al.[212]. In addition, considerable data support the notion that the increase in FoxO1 expression in muscle might act as an inhibitor of myogenesis [257-261]. Moreover Wu et al. support the idea that the mTOR pathway promotes myogenesis. Consistent with a negative role for FoxO1 in differentiation, FoxO1 decreases a specific subset of genes in the mTOR signaling pathway, thus inhibiting myogenesis [262] Our results show a decrease in muscle FoxO1 gene expression for the SRL treatment, which might support this hypothesis, even if no differences were observed in the protein amount. Furthermore,

decrease in FoxO1 gene expression was found in adipose tissue, for both treated group, but protein amount seems not changed. Nakae et al. explain the negative role for Foxo1 in adipocyte differentiation, by suppressing progression through the cell cycle, an event required in the early stages of adipose conversion [263]. That might suggest a consequent enhancing in adipocyte differentiation by these drugs.

In addition, some studies have proposed an implication of FoxO1 in hepatic *de novo* lipogenesis and hepatic triglyceride accumulation, presumably through increases in the transcription of SREBP1-c [264-266]. According to these findings, with the CsA and SRL treatments we observed a stimulation of hepatic lipogenesis through the SREBP-1 pathways, in terms of both gene and protein expression, with its isoform 1c being significantly expressed in liver after treatment with either drug. SREBP1-c is a major transcription factor that stimulates expression of genes involved in fatty acid synthesis [267]. SREBP1-c gene expression was also over expressed in muscle of CsA treated rats, but protein amount wasn't.

Iizuka et al. proposed a similar mechanism between SREBP-1 and ChREBP; they seem to regulate different steps in glycolysis and gluconeogenesis, and to share genes involved in lipogenesis. In detail, high glucose levels can lead to ChREBP activation, which in turn, activates glycolitic genes, such as G6P and lipogenic gene expression, such as ACC [268]. As a result, we have also assessed ChREBP gene and protein expression. observing an increase in ChREBP gene expression in liver for the SRL treated group. A possible explanation could be the increased glucose levels observed in our SRL treated group, this might lead to an up regulation in ChREBP gene expression. Conversely, the down regulation in ChREBP gene expression in fat could reflect the inhibition in lipogenesis by SRL.

Finally, ACC1 gene and protein expression was assessed in order to identify its involvement in the impaired lipide profile observed by CsA and SRL. ACC1, an isoform of ACC, that catalyzes the irreversible reaction of fatty acid synthesis by carboxylating acetyl CoA to produce malonyl-CoA [269]. In our study ACC1 gene expression showed no significant changes in liver, while the protein amount tended to be increased for the CsA treatment, suggesting an enhanced lipolysis. Forthermore, CsA treatment caused down

regulated ACC1 gene expression in fat, showing impaired lipolysis in this tissue, in accordance with our results. Importantly, ACC1 gene expression was increased in muscle for the CsA treated group. During active lipogenesis, the pool of malonyl-CoA, produced from the ACC reaction, increases and inhibits carnitine palmitoyltransferase I (CPT I), resulting in an inhibition of β oxidation pathway [31]. However we should point out that, the predominant form in muscle is ACC2, it is recognized that ACC1 is also expressed in this tissue, and its contribution should be considered [31]. These results demonstrate that CsA may be involved in inhibiting fatty acid oxidation in muscle and the consequent lipid accumulation [269].

Taken together, these results indicate that there are glucogenic and lipogenic effects of the studied IAs on hepatocytes and adipocytes. Adipocytes of treated rats show a reduced lipogenesis and an increased lipolysis, as previously demonstrated [249, 231, 254], while hepatic lipogenesis and gluconeogenesis seem to increase. This can lead to elevated circulating fatty acids and glycerol and this might lead to fatty acid deposition as ectopic triglycerides in the insulin target tissues, such as liver and skeletal muscle, as suggested by Roden et al. [270]. In addition, glycerol is an important substrate for hepatic gluconeogenesis directly contributing to glucose production [271].

Chapter V. Conclusions

V - Conclusions

NODAT is a common complication after solid organ transplantation and has variably been reported to have an adverse impact on patient and allograft outcomes.

Adverse effects including dyslipidemia and glucose intolerance are extremely common after transplantation and contribute significantly to cardiovascular morbidity and mortality.

In the present study it is shown that both CsA and SRL, act in adipose tissue inhibiting glucose uptake, enhancing lipolysis, stimulating and attenuating lipogenesis partially via down-regulation of lipogenic genes. Additionally, these drugs stimulate gluconeogenesis and lipogenesis in liver, while they may be involved in decreased fatty acid oxidation in muscle. In comparison with CsA, sirolimus seems to have a more pronounced effect on glucose intolerance, as well as on genes involved in the regulation of lipid metabolism.

Hence, the observed findings, caused by IA treatments with concentrations that are commonly present in the circulation of treated patients may provide one explanation for the insulin resistance and the development of NODAT during immunosuppressive therapy.

Chapter VI. References

VI – References

1. Powers, A.C. (2008). “Diabetes Mellitus” Nat Clin Pract Endocrinol Metab. 4(12): 664-665.

2. Saltiel, A.R.; C.R. Kahn (2001) “Insulin signalling and the regulation of glucose and lipid metabolism” Nature 414(6865): 799-806.

3. Rizza R.A.; Mandarino, L.J.; Gerich, J.E. (1981) “Dose-response characteristics for effects of insulin on production and utilization of glucose in man” Am J Physiol Endocrinol Metab. 240(6): E630-E639.

4. Taguchi, A.; M.F. White (2008) “Insulin-like signaling, nutrient homeostasis, and life span” Annu Rev Physiol 70(1): 191-212.

5. Bouche, C.; et al. (2004) “The cellular fate of glucose and Its relevance in type 2 diabetes” Endocr. Rev. 25(5): 807-830.

6. De Meyts, P.; Whittaker, J. (2002) “Structural biology of insulin and IGF1 receptors: implications for drug design” Nat. Rev. Drug Discov. 1(10): 769-783.

7. Belfiore, A.; et al. (2009) “Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease” Endocr. Rev. 30(6): 586-623.

8. Patti, M.E.; Kahn, C.R. (1998) “The insulin receptor - a critical link in glucose homeostasis and insulin action” J. Basic Clin. Physiol. Pharmacol. 9(2): 89-109.

9. Taniguchi, C.M.; Ueki, K.; Kahn, C.R. (2005) “Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism” J. Clin. Invest. 115(3): 718-727.

10. Lizcano, J.M.; Alessi, D.R. (2002) "The insulin signalling pathway." Curr. Biol. 12(7): R236-R238.

11. Taniguchi, C.M.; Emanuelli, B.; Kahn C.R. (2006). "Critical nodes in signalling pathways: insights into insulin action" Nat. Rev. Mol. Cell. Biol. 7(2): 85-96.

12. Cheng, Z., Tseng, Y.; White M.F. (2010) "Insulin signaling meets mitochondria in metabolism." Trends. Endocrinol. Metab. 21(10): 589-598.

13. Ishikura, S.; Koshkina, A.; Klip, A (2008) “Small G proteins in insulin action: Rab and Rho families at the crossroads of signal transduction and GLUT4 vesicle traffic” Acta Physiologica 192(1): 61–74.

14. Laplante, M.; Sabatini, D.M. (2009) “An emerging role of mTOR in lipid biosynthesis” Curr. Biol. 19(22): R1046-R1052.

15. Cheng, Z., S. Guo, et al. (2009) “Foxo1 integrates insulin signaling with mitochondrial function in the liver” Nat. Med. 15(11): 1307-1311.

16. Cell Signaling Technology, I. (2011). "Insulin receptor signaling." Available at: https://www.cellsignal.com/pathways/glucose-metabolism.jsp Accessed May 2012.

17. Altomonte, J., et al. (2003) “Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice” Am. J. Physiol. Endocrinol. Metab. 285(4): E718-28.

18. Puigserver, P.; et al.(2003) “Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction” Nature 423(6939): 550-555.

19. Lehmann, O.J.; et al. (2003) “Fox's in development and disease” Trends Genet. 19: 339-344.

20. Nakae, J.; et al. (2002) “Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1” Nat. Genet. 32: 245-253.

21. Cade, W.T. (2008) “Diabetes-Related Microvascular and Macrovascular Diseases in the Physical Therapy Setting” Phys. Ther. 88(11): 1322-35.

22. King, M.W. (1996) “Gluconeogenesis: Glucose Synthesis” Available at: themedicalbiochemistrypage.org Accessed March 2012.

23. Bouché, C.; Serdy, S.; Kahn, C.R.; Goldfine, A.B. (2004) “The cellular fate of glucose and its relevance in type 2 diabetes” Endocr Rev 25(5):807-30.

24. Finn, P.F.; Dice, J.F. (2006) “Proteolytic and lipolytic responses to starvation” Nature 22(7): 830–844.

25. Puigserver, P.; Spiegelman, B.M. (2003) "Peroxisome proliferator-activated receptor-γ Coactivator 1α (PGC-1α): Transcriptional coactivator and metabolic regulator" Endocr. Rev. 24(1): 78-90.

26. Kersten, S. (2001) “Mechanisms of nutritional and hormonal regulation of lipogenesis” EMBO Rep.2(4): 282–6.

27. Ferré, P.; Foufelle, F. (2010) “Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c.” Diabetes Obes. Metab. 12: 83-92.

28. Moon, Y.A.; et al. (2012) “The Scap/SREBP pathway is essential for developing diabetic fatty liver and carbohydrate-induced hypertriglyceridemia in animals” Cell Metab. 15(2): 240-246.

29. Moore, D.D. (2012) “Nuclear receptors reverse McGarry's vicious cycle to insulin resistance”. Cell Metab. 15(5): 615-622.

30. MA, L.; Robinson, N.L.; Towle, H.C. (2006) “ChREBP*Mlx is the principal mediator of glucose-induced gene expression in the liver” J. Biol. Chem. 281(39): 28721-28730.

31. McGarry, J.D. (2002) “Dysregulation of fatty acid metabolism in the etiology of type 2 diabetes” Diabetes 51(1): 7-18.

32. Delgado, T.C.; et al. (2009) “Sources of hepatic triglyceride accumulation during high-fat feeding in the healthy rat” NMR Biomed. 22(3): 310-317.

33. Cao, J. (2011) “Targeting Acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1) with small molecule inhibitors for the treatment of metabolic diseases” J Biol Chem. 286(48):41838-41851.

34. American Diabetes Assosiation (2011). “Diagnosis and classification of diabetes mellitus” Diabetes Care 34: S62-S69.

35. Pickup, J.C.; Williams, G. (2003). “Textbook of diabetes” Oxford: Blackwell Science.

36. Shaw, J.E., Sicree, R.A.; Zimmet, P.Z. (2010) “Global estimates of the prevalence of diabetes for 2010 and 2030” Diabetes Res. Clin. Pract. 87: 4-14.

37. World Health Organization. “Diabetes action now booklet: a life-threatening condition” Available at: www.who.int/diabetes/BOOKLET_HTML/en/index3.html Accessed May 2012.

38. World Health Organization. “Diabetes: the cost of diabetes” Aviable at: http://www.who.int/mediacentre/factsheets/fs236/en/ Accessed May 2012

39. Hogan, P.; Dall, T.;Nikolov, P. (2002) “Economic costs of diabetes in the US in 2002” Diabetes Care 26: 917–932.

40. Hex, N.; et al. (2012) “Estimating the current and future costs of Type 1 and Type 2 diabetes in the UK, including direct health costs and indirect societal and productivity costs” Diabet Med. 29(7): 855-862.

41. Roglic, G.; et al. (2005) “The burden of mortality attributable to diabetes: Realistic estimates for the year 2000”. Diabetes Care 28(9): 2130-2135.

42. “Diabetes in the UK 2011/12: Key Statistics on Diabetes”. London: Diabetes UK, 2011. Available at http://www.diabetes.org.uk/Documents/Reports/Diabetes-in-the-UK-2011-12.pdf Accessed May 2012.

43. Al-Mutairi; et al. (2007) “Genetics of Type 1 Diabetes Mellitus” Kuwait Medical Journal 39(2): 107-115.

44. Hu, F.B. (2012) “Globalization of Diabetes” Diabetes Care 34: 1249–1257.

45. Ensminger, A.H.; et al. (1995). The Concise Encyclopedia of Foods & Nutrition, CRC Press.

46. Dean, L.; McEntyre, J. (2004). “The Genetic Landscape of Diabetes” NCBI Bookshelf.

47. World health Organization (WHO 1999).Diagnosis and classification of diabetes mellitus."Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications" Report of a WHO consultation; pp:14.

48. American Diabetes Assoc. (2011) "Diagnosis and classification of diabetes mellitus." Diabetes Care 34(1): S62-S69.

49. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. (1997) Diabetes Care 20: 1183–1197.

50. Diabetes Prevention Program Research Group (2007) “The prevalence of retinopathy in impaired glucose tolerance and recent-onset diabetes in the Diabetes Prevention Program”. Diabet Med. 24: 137–1344.

51. Cheng Y.L.; Gregg, E.W; Geiss, L.S. (2009) “Association of A1C and fasting plasma glucose levels with diabetic retinopathy prevalence in the U.S. population” Diabetes Care 32: 2027–2032.

52. Alberti, K.G.; Zimmet, P.Z. (1998) “Definition, diagnosis and classification of diabetes mellitus and its complications, Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation” Diabet Med. 15(7): 539–553.

53. Buysschaert, M; Bergman, M.; (2011) “Definition of prediabetes” Med Clin North Am 95(2):289-97.

54. Saad, M.F.; et al. (1988) “The natural history of impaired glucose tolerance in the Pima Indians”. N. Engl. J. Med. 319(23): 1500-1506.

55. Meigs, J.; et al. (2003) “The natural history of progression from normal glucose tolerance to type 2 diabetes in the Baltimore Longitudinal Study of Aging” Diabetes 52(6): 1475-1484.

56. Abdul-Ghani, M; DeFronzo, R. (2009) “Pathophysiology of prediabetes” Curr. Diabetes Report 9(3): 193-199.

57. Aekplakorn, W.; et al. (2003). “The prevalence and management of diabetes in Thai adults”. Diabetes Care. 26(10): 2758-2763.

58. Dirice, E.; Kulkarni, R.N. (2011). “Pathways underlying β-cell regeneration in type 1, type 2 and gestational diabetes- Islet Cell Growth Factors” Landies Bioscience Texas.

59. William, C. , “Diabetes mellitus” Available at: http://www.medicinenet.com/diabetes_mellitus/article.htm Accessed May 2012.

60. "Diabetes Mellitus (DM): Diabetes Mellitus and Disorders of Carbohydrate Metabolism: Merck Manual Professional". Merck.com.

61. Karvonen, M; et al. (2000) “Incidence of Childhood Type 1 DiabetesWorldwide” Diabetes Care 23: 1516–1526.

62. Karvonen, M.; et al. (1993) “A review of the recent epidemiological data on the worldwide incidence of type 1 (insulin-dependent) diabetes mellitus” Diabetologia 36: 883–892.

63. Diabetes, O. N. d. (2009). “Programa Nacional de Prevenção e Controlo da Diabetes 2008-2017. Diabetes: factos e números” Observatório Nacional da Diabetes, Portugal.

64. Karvonen, M. et al. (1997) “Sex difference in the incidence of insulin-dependent diabetes mellitus: an analysis of the recent epidemiological data” Diabetes Metab. Rev. 13: 275–291.

65. Pirot, P.; et al.; (2008). "Mediators and mechanisms of pancreatic β-cell death in type 1 diabetes." Arq. Bras. Endocrinol. Metabol. 52: 156-165.

66. Dotta, F.; et al. (2007) "Coxsackie B4 virus infection of β-cells and natural killer cell insulitis in recent-onset type 1 diabetic patients." Proc. Nat. Acad. Sci. 104(12): 5115-5120.

67. International Diabetes Federation, 2011, “About Diabetes”. Available at http://www.idf.org Accessed March 2012

68. Stern, M.P. (1995) “Diabetes and cardiovascular disease: the common soil hypothesis” Diabetes 44: 369–381.

69. Noble, D.; et al. (2011)“Risk models and scores for type 2 diabetes: systematic Review” BMJ 343: d7163.

70. Statistics Canada Percentage reporting a diagnosis of diabetes, by age group and sex, household population aged 12 or older, Canada.Ottawa, ON: Statistics Canada; 2008. Available at: www.statcan.gc.ca. Accessed May.2012.

71. Ohinmaa, A.; Jacobs, P.; Simpson, S.; Johnson, J.A. (2004) “The projection of prevalence and cost of diabetes in Canada: 2000 to 2016” Can. J. Diabetes. 28(2): 1–8.

72. Carvalho, E.; Eliasson, B.; Wesslau, C.; Smith, U. (2000) “Impaired phosphorylation and insulin-stimulated translocation to the plasma membrane of protein kinase B/Akt in adipocytes from Type II diabetic subjects” Diabetologia 43(9): 1107-1115.

73. Pederson, T.M.; et al. (2001) "Serine/threonine phosphorylation of IRS1 triggers its degradation" Diabetes 50(1) 24-31.

74. Gual, P.; et al. (2005) "Positive and negative regulation of insulin signaling through IRS-1 phosphorylation" Biochimie 87(1): 99-109.

75. Jeong, K.; Yongzhong, W.; Sowers, J.R. (2008) “Role of Mitochondrial Dysfunction in Insulin Resistance” Circ. Res. 102(4): 401-414.

76. Petersen, K.F.; et al. (2004). "Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes" N. Engl. J. Med. 350(7): 664-671.

77. Patti, M.-E.; Corvera, S.; (2010) "The role of mitochondria in the pathogenesis of type 2 diabetes." Endocr Rev 31(3): 364-395.

78. Yuan, M.; et al. (2001). "Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkβ" Science 293(5535): 1673-1677.

79. Sheng, T.; Yang, K. (2008) "Adiponectin and its association with insulin resistance and type 2 diabetes" J. Genet. Genomics 35(6): 321-326.

80. Yang, Q.; et al. (2005). "Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes" Nature 436(7049): 356-362.

81. Steppan, C.M.; et al. (2001). "The hormone resistin links obesity to diabetes." Nature 409(6818): 307-312.

82. Galic, S.; et al. (2010). "Adipose tissue as an endocrine organ" Mol. Cell Endocrinol. 316(2): 129-139.

83. Fernández-Morera, J.L.; Rodríguez-Rodero, S.; Menéndez-Torre, E; Fraga, M.F. (2010). "The possible role of epigenetics in gestational diabetes: cause, consequence, or both." Obstet. Gynecol. Int. 2010:605163.

84. Jovanovic, L.; et al., (2005) "Elevated pregnancy losses at high and low extremes of maternal glucose in early normal and diabetic pregnancy: evidence for a protective adaptation in diabetes," Diabetes Care 28(5): 1113-1117.

85. Hoffman, L. (1998) "Gestational diabetes mellitus (GDM)," Med. J. Aust. 168(3): 140-146.

86. Getahun, D.; et al. (2008) "Gestational diabetes in the United States: temporal trends 1989 through 2004," Am. J. Obstet Gynecol. 198(5): 525-530.

87. Vrachniset, N.; et al. (2012) "Previous Gestational DiabetesMellitus and Markers of Cardiovascular Risk" Int. J. Endocrinol. 2012: 458610.

88. Lambrinoudaki, I.; Vlachou, S.A.; Creatsas, G. (2010) "Genetics in gestational diabetes mellitus: association with incidence, severity, pregnancy outcome and response to treatment" Curr. diabetes reviews 6(6): 393-399.

89. Fox, K.M.; Rodbard, H.W.; Green, A.J.; Grandy, S.(2009) "Trends in method of diagnosis of type 2 diabetes mellitus: results from SHIELD" Inter. J. Endocrinol. 2009:796206.

90. Stone, C.A.; et al. (2002). “Gestational diabetes in Victoria in 1996: incidence, risk factors and outcomes” Med. J. Aust. 177(9): 486-491.

91. Petry, C.J. (2010) “Gestational diabetes: risk factors and recent advances in its genetics and treatment,” Br J Nutr. 104(6): 775-787.

92. Vohr, B.R.; Boney, C.M. (2008) “Gestational diabetes: the forerunner for the development of maternal and childhood obesity and metabolic syndrome?” J Matern Fetal Neonatal Med. 21(3): 149-157.

93. Catalano, P.M., et al. (1993) "Carbohydrate metabolism during pregnancy in control subjects and women with gestational diabetes" Am. J. Physiol. Endocrinol. Metab. 264(1): E60-E67.

94. Homko, C.; et al. (2001). "Insulin ecretion during and after pregnancy in patients with gestational diabetes mellitus" J. Clin. Endocrinol. Metab. 86(2): 568-573.

95. Barbour, L. A.; et al. (2007) "Cellular mechanisms for insulin resistance in normal pregnancy and gestational diabetes" Diabetes Care 30(2): S112-S119.

96. Buchanan, T.A.; Xiang, A.H. (2005). "Gestational diabetes mellitus" J. Clin. Invest. 115(3): 485-491.

97. Hara, K.; Yamauchi, T.; Kadowaki, T. (2005) “Adiponectin: an adipokine linking adipocytes and type 2 diabetes in humans” Curr. Diab. Rep. 5:135-140.

98. Harlev, A.; Wiznitzer, A. (2010) "New insights on glucose pathophysiology in gestational diabetes and insulin resistance" Curr. Diab. Rep. 10(3): 242-247.

99. Worda, C.; et al. (2004) “Decreased plasma adiponectin concentrations in women with gestational diabetes mellitus” Am. J. Obstet. Gynecol. 191: 2120–2124.

100. Megia, A. (2008) "Insulin sensitivity and resistin levels in gestational diabetes mellitus and after parturition" Eur. J. Endocrinol. 158(2): 173-178.

101. Catalano P.M.; et al. (2002) “Downregulated IRS-1 and PPARgamma in obese women with gestational diabetes: relationship to FFA during pregnancy” Am. J. Physiol. Endocrinol. Metab. 282:E522–E533.

102. Center for Desease Control and Prevention, National Diabetes Fact Sheet, 2011, Available at: http://www.cdc.gov/diabetes/pubs/factsheet11.htm Accessed in May 1 2012

103. Scherer M.N.; et al. (2007) “Current concepts and perspectives of immunosuppression in organ transplantation” Langenbecks Arch Surg 392:511-523.

104. Cole, E.H.; Johnston, O.; Rose, C.L.; Gill, J.S. (2008) “Impact of acute rejection and new-onset diabetes on long-term transplant graft and patient survival” Clin. J. Am. Soc. Nephrol. 3: 814-821.

105. Massy, Z.A. (2001) “Hyperlipidemia and cardiovascular disease after organ transplantation” Transplantation 72: S13-15.

106. Bodziak, K.A.; Hricik, D.E. (2009) “New-onset diabetes mellitus after solid organ transplantation” Trasplant Int. 22: 519-530.

107. Perrea, D.N.; et al. (2008) “Correlation between lipid abnormalities and immunosuppressive therapy in renal transplant recipients with stable renal function” Int. Urol. Nephrol. 40: 521-527.

108. Markell, M.;(2004) “New-onset diabetes mellitus in transplant patients: pathogenesis, complications, and management”. Am. J. Kidney Dis. 43(6): 953-965.

109. Salvadori, M.; Bertoni, E.; Rosati, A.; Zanazzi, M. (2003) “Post-transplant diabetes mellitus”. J. Nephrol. 16(5): 626-634.

110. Jindal, R.M. (1994) “Post-transplant diabetes mellitus- a review”. Transplantation 58(12): 1289-1298.

111. Reisæter, A.V.; Hartmann, A. (2001) “Risk factors and incidence of posttransplant diabetes mellitus”. Trans. Proc. 33(5): S8-S18.

112. Mora, P.F. (2005) “Post-transplantation diabetes mellitus” Am. J. Med. Sci. 329(2): 86-94.

113. Pham, P.T.; et al. (2001) “New onset diabetes after transplantation(NODAT): an overview” Diabetes Metab Syndr Obes. 4: 175-186.

114. Davidson, J. et al. (2003) “New-onset diabetes after transplantation: 2003 International Consensus Guidelines” Transplantation 7: SS3–SS24.

115. Baid, S.; et al. (2001) “Posttransplant diabetes mellitus in liver transplant recipients: risk factors, temporal relationship with hepatitis C virus allograft hepatitis, and impact on mortality” Transplantation. 72: 1066–1072.

116. Knobler, H.; et al. (1998) “Higher incidence of diabetes in liver transplant recipients with hepatitis C” J. Clin. Gastroenterol. 26: 30–33.

117. Ye, X.; Kuo, H.-T.; Sampaio, M.S.; Jiang, Y.; Bunnapradest, S.; (2010) “Risk factors for the development of new-onset diabetes mellitus after transplant in adult lung transplant recipients”. Clin Transplant. 1111: 1-7.

118. Sulanc, E.; et al. (2005) “New-onset diabetes after kidney transplantation: an application of 2003 International Guidelines”. Transplantation 80(7):945–952.

119. Durrbach, A. (2006) “Diabetes after transplantation” Abstract Nephrol. Ther. 3:S197-199.

120. (No authors listed) (2011) “Post-transplantational diabetes mellitus”; Klin. Med. 89(5): 16-20.

121. Jodi, M.; et al. (2003) “Current immunosuppressive agents: efficacy, side effects, and utilization” Pediatr. Clin. N. Am. 50: 1283-1300.

122. Egidi, F. M. (2005) “Management of Hyperglycaemia After Pancreas Transplantation: Are New Immunosuppressants the Answer?” Drugs 65(2): 153-166.

123. Morrisett, J.D.; et al. (2002) “Effects of sirolimus on plasma lipids, lipoprotein levels, and fatty acid metabolism in renal transplant patients” J. Lip. Res 43(8): 1170-1178.

124. Chow, K.M.; Li, P.K; (2008) “Review article: New-onset diabetes after transplantation” Nephrology 13(8):737-744.

125. Fernandez, L.A.; et al. (1999) “The effects of maintenance doses of FK506 versus cyclosporin A on glucose and lipid metabolism after orthotopic liver transplantation” Transplantation 68(10): 1532-1541.

126. Ingle D. (1941) “The production of glycosuria in the normal rat by means of 17-hydroxy-11-dehydrocorticosterone”. Endocrinology 29: 649-652.

127. Rizza, R.A.; Mandarino, L.J.; Gerich, J.E. (1982) “Cortisol-induced insulin resistance in man: impaired suppression of glucose production and stimulation of glucose utilization due to a postreceptor detect of insulin action” J. Clin. Endocrinol. Metab. 54(1): 131-138.

128. Bressler, P;.DeFronzo, R.A.; ()1994 “Drugs and diabetes” Diabetes Ver. 2: 53-84.

129. Leahy J.L.;et al. (2000) “Medical management of diabetes mellitus - Drug-induced disorders of glucose metabolism” Marcel DeckerNew York.

130. Miller, L.W. (2002) “Cardiovascular toxicities of immunosuppressive agents”. Am. J. Transplant 2(9): 807-818.

131. Kobashigawa, J.A.; Kasiske, B.L. (1997) “Hyperlipidemia in solid organ transplantation”. Transplantation 63: 331–338.

132. Blum, A.; Aravot, D. (1996) “Heart transplantation – an update” Clin. Cardiol. 19: 930–938.

133. Øzbay, L.A.; et al. (2011) “Cyclosporin and tacrolimus impair insulin secretion and transcriptional regulation in INS-1E beta-cells” Brit. J. Pharm. 162:136–146.

134. Fruman, D.A.; Klee, C.B.; Bierer, B.E.; Burakoff, S.J. (1992) “Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A” Proc. Natl. Acad. Sci. U. S. A. 89: 3686-3690.

135. Liu, J; et al. (1991) “Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes” Cell 66: 807-815.

136. Sugimoto, T; Stewart, S.; Guan, K.L. (1997) “The calcium/calmodulin-dependent protein phosphatase calcineurin is the major Elk-1 phosphatase” J. Biol. Chem. 272: 29415-29418.

137. Cho, C.S.; et al. (2003) “Modulation of the electrophoretic mobility of the linker for activation of T cells (LAT) by the calcineurin inhibitors CsA and FK506: LAT is a potential substrate for PKC and calcineurin signaling pathways” Cell Signal 15: 85-93.

138. Faul, C.; et al. (2008) “The actin cytoskeleton of kidney podocytes is a direct target of the antiproteinuric effect of cyclosporine A” Nat. Med. 14: 931-938.

139. Nielsen, J.H.; Mandrup-Poulsen, T.; Nerup, J. (1986) “Direct effects of cyclosporin A on human pancreatic beta-cells” Diabetes 35: 1049–1052.

140. Paty, B.W.; Harmon, J.S.; Marsh, C.L.; Robertson, R.P. (2002). “Inhibitory effects of immunosuppressive drugs on insulin secretion from HIT-T15 cells and wistar rat islets” Transplantation 73(3): 353-357.

141. Redmon, J.B.; et al. (1996) “Effects of tacrolimusrolimus (FK506) on human insulin gene expression, insulin mRNA levels, and insulin secretion in HIT-T15 cells” J. Clin. Invest. 98: 2786–2793.

142. Uchizono, Y.et al. (2004) “Tacrolimus impairment of insulin secretion in isolated rat islets occurs at multiple distal sites in stimulus-secretion coupling” Endocrinology 145: 2264–2272.

143. Lawrence, M.C.; Bhatt, H.S.; Easom, R.A. (2002) “NFAT regulates insulin gene promoter activity in response to synergistic pathways induced by glucose and glucagon-like peptide-1” Diabetes 51: 691-698.

144. Oetjen, E.; et al. (2003) “Inhibition of human insulin gene transcription by the immunosuppressive drugs cyclosporin A and tacrolimusrolimus in primary, mature islets of transgenic mice” Mol. Pharmacol. 63: 1289-1295.

145. Heit, J.J.; et al. (2006) “Calcineurin/NFAT signaling regulates pancreatic beta-cell growth and function” Nature 443: 345-349.

146. Lawrence, M.C.; McGlynn, K.; Park, B.H.; Cobb, M.H. (2005) “ERK1/2-dependent activation of transcription factors required for acute and chronic effects of glucose on the insulin gene promoter” J. Biol. Chem. 280: 26751-26759.

147. Ranta, F.; et al. (2008) “Regulation of calcineurin activity in insulin-secreting cells: stimulation by Hsp90 during glucocorticoid-induced apoptosis” Cell Signal 20: 1780–1786.

148. Ebihara K.; et al. (1996) “Cyclosporin A stimulation of glucose-induced insulin secretion in MIN6 cells” Endocrinology 137: 5255-5263.

149. Su, Q. et al (1995) “Nephrotoxicity of cyclosporin A and FK506: inhibition of calcineurin phosphatase” Ren. Physiol. Biochem. 18: 128-139.

150. Weir, M.R., Fink, J.C. (1999) “Risk for posttransplant Diabetes mellitus with current immunosuppressive medications” Am. J. Kidney Dis. 34: 1-13.

151. Roland, M.; et al. (2008) “Immunosuppressive medications, clinical and metabolic parameters in new-onset diabetes mellitus after kidney transplantation” Transpl. Int. 21: 523-530.

152. Subramanian, S.; Trence, D.L. (2007) “Source Immunosuppressive agents: effects on glucose and lipid metabolism” Endocrinol. Metab. Clin. North Am. 36(4): 891-905.

153. Laupacis, A.; et al. (1981) “Hyperbilirubinaemia and cyclosporin-A levels”. Lancet. 2: 1426-1427.

154. Borel, J.F.; et al. (1976) “Bilogical effects of Cyclosporin A: a new antilymphocytic agent”. Agents. Action. 6: 468-475.

155. Faulds, D.; Goa, K.L.; Benfield, P. (1993) “Cyclosporin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in immunoregulatory disorders” Drugs 45: 953-1040.

156. Atkinson, A.; et al. (1983) “Blood and and tissue distribution of cyclosporine in humans and mice” Transp. Proc. 15: 2430-2436.

157. Miraz, W; Zink, R.A.; Graf, A. (1983) “Distribution and Transfer of Cyclosporin among the various human lipoprotein classes” Transplant Proc. 15: 2426:2431.

158. Ryffel, B.; et al. (1988) “Biological significance of ciclosporin metabolities” Transplant Proc. 20: 6575-580.

159. Khanna, A.; Li, B.; Stenzel, K.H.; Suthanthiran, M. (1994) “Regulation of new DNA synthesis in mammalian cells by cyclosporine. Demonstration of a transforming growth factor beta-dependent mechanism of inhibition cell growth” Transplantation 57: 577-582.

160. Mihatsch, M.J.; et al. (1998) “The side effects of cyclosporine-A and tacrolimus” Clin. Nephrol. 49: 356-63.

161. Citterio, F., Kahan, B.D. (1989) “The inhibitory effect of cyclosporine on the nuclear proliferative response to a variety of T cells activators” Transplatation 47: 334-338.

162. Hess, A.D.; Esa, A.H.; Colombani, P.M. (1998) “Mechanism of action of cyclosporine : effects on cells of the immune system and on subcellular events in T cells activation” Transplant Proc. 20(2): 29-40.

163. King, M.; Macklem, P.T. (1997) “Rheological properties of microliter quantities of normal mucous” J. Appl. Phisiol. 42: 797-802.

164. Sehgal, S.N. (1998) “Rapamune (RAPA, rapamycin, sirolimus): Mechanism of action immunosuppressive effect results from blockade of signal transduction and inhibition of cell cycle progression” Clin Biochem 31(5): 335-340.

165. Terada, N.; et al. (1993) “Rapamycin blocks cell cycle progression of activated T cells prior to events characteristic of the middle to late G1 phase of the cycle” J. Cell. Physiol. 154(1):157.

166. Andoh, T.F.; Burdmann, E.A.; Bennett, W.M.; (1997) “Nephrotoxicity of immunosuppressive drugs: experimental and clinical observations” Semin. Nephrol.17: 34-39.

167. Golbaekdal, K.; Nielsen, C.B.; Djurhuus, J.C.; Pederson, E.B (1994) “Effects of rapamycin on renal hemodynamics, water and sodium excretions and plasma levels of angiotensin II, aldosterone, atrial natriuretic peptide and vasopressin in pigs” Transplantation 58: 1153-1157.

168. Campistol, J.M.; et al. (2006) “Sirolimus therapy after early cyclosporine withdrawal reduces the risk for cancer in adult renal transplantation” J. Am. Soc. Nephrol. 17: 581-589.

169. Oberbauer, R.; Segoloni, G.; Campistol, J.M. (2005) “Early cyclosporine withdrawal from a sirolimus-based regimen results in better renal allograft survival and renal function at 48 months after transplantation”. Transpl. Int. 18: 22-26.

170. Sehgal, S.N. (1995) “Rapamune (sirolimus, rapamycin): an overview and mechanism of action” Ther. Drug Monitor 17(6): 660-665.

171. Brown, E.J.; et al. (1994) “A mammalian protein targeted by G1-arresting rapamycin-receptor complex” Nature, 369: 756-758.

172. Hay, N.; Sonnenberg, N.; (2004) “Upstream and downstream of mTOR” Genes Dev. 18: 1926–1945.

173. Jaeschkle, A.; Dennis, P.B.; Thomas, G. (2004) “mTor a mediator of intracellular homeostasis” Curr Top Microbiol Immunol, 279: 283–298.

174. Aggarwal, D.; Fernandez, M.L.; Soliman, G.A. (2006) “Rapamycin,an mTOR inhibitor, disrupts triglyceride metabolism in guinea pigs” 5(6): 794-802.

175. Hoogeven, R.C.; et al. (2001) “Effect of sirolimus on the metabolism of apoB100-containing lipoproteins in renal transplant patients” Transplantation 72 1244–1250.

176. http://www.landesbioscience.com/curie/images/chapters/Grinyo1color.jpg. Accessed June 2012.

177. Sipula, I.J.; Brown, N.F.; Perdomo, G. (2006) "Rapamycin-mediated inhibition of mammalian target of rapamycin in skeletal muscle cells reduces glucose utilization and increases fatty acid oxidation" Metabolism 55(12): 1637-1644.

178. Cho, H.J.; Park, J.; Lee, H.W. (2004) "Regulation of adipocyte differentiation and insulin action with rapamycin" Biochem. Biophys. Res. Commun. 321(4): 942-948.

179. Berg, C.E.; Lavan, B.E.; Rondinone, C.M. (2002) "Rapamycin partially prevents insulin resistance induced by chronic insulin treatment" Biochem. Biophys. Res. Commun. 293(3): 1021-1027.

180. Rosendal, F. et al. (2005) "Does Chronic Low-Dose Treatment With Cyclosporine Influence the Brain? A Histopatological Study in Pigs" Transplantation Proceedings 37: 3305-3308.

181. Oriji, G.K.; Schanz, N. (2001) "Nitric Oxide in CsA-induced Hypertension: role of beta-adrenoceptor antagonist and thromboxane A2" Prostaglandins Leukot. Essent. Fatty Acids 65(5-6): 259-263

182. Ost, L. (1987) "Impairment of prednisolone metabolism by cyclosporine treatment in renal graft recipients" Transplantation 44(4): 533-535.

183. Hjelmesaeth, J.; et al. (2007) "The impact of short-term cyclosporin A treatment on insulin secretion and insulin sensitivity in man". Nephrol. Dial. Transplant 22(6): 1743-1749.

184. Brown, J.H.; et al. (1997) "Influence of immunosuppressive therapy on lipoprotein(a) and other lipoproteins following renal transplantation". Nephron. 75(3): 277-282.

185. Mayer, A.D.; et al. (1997) "Multicenter randomized trial comparing tacrolimus (FK506) and cyclosporine in the prevention of renal allograft rejection". Transplantation 64: 436-441.

186. Bennett, W.M.; et al. (1996) “Chronic cyclosporine nephropathy: the Achilles' heel of immunosuppressive therapy”. Kidney Int. 50: 1089-1100.

187. Kasiske, B.L.; et al. (2003) “Diabetes mellitus after kidney transplantation in the United States”. Am. J. Transplant 3(2): 178-185.

188. Walczak, D.A.; et al. (2005) “Increased risk of post-transplant diabetes mellitus despite early steroid discontinuation in Hispanic kidney transplant recipients” Clin. Transplant 19(4): 527-531.

189. Rhen, T.; Cidlowski, J.A.; (2005) “Anti-inflammatory action of glucocorticoids—new mechanisms for old drugs”. N. Engl. J. Med. 353(16): 1711-1723.

190. Mathe, D.; et al. (1992) “Prevalence of dyslipidemia in liver transplant recipients”. Transplantation 54(1): 167-170.

191. Kun, L.; et al. (2007) “Sirolimus Modifies Cholesterol Homeostasis in Hepatic Cells: A Potential Molecular Mechanism for Sirolimus-Associated Dyslipidemia” Transplantation 84(8) :1029-1036.

192. Kahan, B.D.; et al. (2005) “Low incidence of malignancy among sirolimus/cyclosporine-treated renal transplant recipients” Transplantation 80(6): 749-758.

193. Taha, C.; et al. (1999) “Opposite translational control of GLUT1 and GLUT4 glucose transporter mRNAs in response to insulin. Role of mammalian target of rapamycin, protein kinase b, and phosphatidylinositol 3-kinase in GLUT1 mRNA translation” J. Biol. Chem. 274(46): 33085-33091.

194. Spinelli, G.A.; et al. (2006) “Relationship of cyclosporin and sirolimus blood concentrations regarding the incidence and severity of hyperlipidemia after kidney transplantation” Braz. J. Med. Biol. Res. 39(1): 19-30.

195. Schenck, F.X; et al. (1991) “Pediatric renal transplantation under FK-506 immunossupression”. J. Urol. 147: 1585-1587.

196. Stepkowski, S.M.; et al. (1997) “Synergistic mechanisms by which Sirolimus and cyclosporin inhibit rat heart and kidney allograft rejection” Clin. Exp. Immunol. 108: 63-68.

197. Klawitter, J.; et al. (2009) “Urine Metabolites Reflect Time-Dependent Effects of Cyclosporine and Sirolimus on Rat Kidney Function” Chem Res Toxicol. 22(1): 118–128.

198. Groth, C.G.; et al. (1999) “Sirolimus (rapamycin)-based therapy in human renal transplantation: similar Efficacy and Different Toxicity Compared with Cyclosporine” Clinical Transplantation 67(7): 1036-1042

199. Carvalho, E; et al. (20001) “Insulin resistance with low cellular IRS-1 expression is also associated with low GLUT-4 expression and impaired insulin-stimulated glucose transport” FASEB J. 15(6): 1101-1103.

200. Carvalho, E.; Rondinone, C.; Smith, U. (2000) “Insulin resistance in fat cells from obese Zucker rats – Evidence for an impaired activation and translocation of protein kinase B and glucose transporter 4” Mol. Cell Biochem. 206: 7-16.

201. Pereira, M.J.; et al. (2012) “mTOR inhibition with rapamycin causes impaired insulin signaling and glucose uptake in human subcutaneous and omental adipocytes” Mol. Cell Endocrinol. 355(1): 96-105.

202. Kashiwagi, A. et al. (1983) “In vitro insulin resistance of human adipocytes isolated from subjects with noninsulin-dependent diabetes mellitus” J. Clin. Invest. 72(4): 1246-1254.

203. Yu, Z.W.; et al. (1997) “Effects of peroxovanadate and insulin in NIDDM adipocytes” Diabetologia. 40(10): 1197-1203.

204. Smith, U.; Sjostrom, L.; Bjornstorp, P. (1972) “Comparison of two methods for determining human adipose cell size” J. Lipid. Res. 13: 822-824

205. Pierce (2009). Thermo Scientific Pierce Protein Assay Technical Handbook Thermo Scientific Inc. USA

206. Rovira, J.; et al. (2008) “Effect of mTOR inhibitor on body weight: from an experimental rat model to human transplant patients” Transpl. Int. 21(10): 992-8.

207. Chang, G.R.; et al. (2009) “Long-term Administration of Rapamycin Reduces Adiposity, but Impairs Glucose Tolerance in High-Fat Diet-fed KK/HlJ Mice” Basic Clin. Pharmacol. Toxicol. 105(3): 188-198.

208. Deblon, N.; et al. (2012) “Chronic mTOR inhibition by rapamycin induces muscle insulin resistance despite weight loss in rats” Br. J. Pharmacol. 165(7): 2325-1240.

209. Gingras, A.C.; Raught, B.; Sonenberg, N. (2001) “Regulation of translation initiation by FRAP/mTOR” Genes Dev. 15(7) :807-826.

210. Wang, X.; Proud, C.G. (2006) “The mTOR pathway in the control of protein synthesis” Physiology 21: 362-9.

211. Sarbassov, D.D.; Ali, S.M.; Sabatini, D.M. (2005) “Growing roles for the mTOR pathway” Curr. Opin. Cell Biol. 17(6): 596-603.

212. Houde, V.P.; et al. (2010) “Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue” Diabetes 59(6): 1338-1348.

213. Marcelli-Tourvieille, S.; et al. (2007) “In Vivo and In Vitro Effect of Sirolimus on Insulin Secretion” Transplantation 83: 532-538.

214. Cha, B.Y.; et al. (1989) “Effects of cyclosporin A on insulin binding and action in isolated fat cells of rat” Korean J. Intern. Med. 4(2): 142-147.

215. Menegazzo, L.A.; et al. (1998) “Mechanism of the diabetogenic action of cyclosporin A” Horm. Metab. Res. 30: 663-667.

216. David-Neto, E.; et al. (2007) “The Dynamics of Glucose Metabolism Under Calcineurin Inhibitors in the First Year After Renal Transplantation in Nonobese Patients” Transplantation 84(1): 50-55.

217. Drachenberg, C.B.; et al. (1999) “Islet cell damage associated with tacrolimus and cyclosporine: morphological features in pancreas allograft biopsies and clinical correlation” Transplantation 68: 396–402.

218. Oetjen, E.; et al. (2003) “Inhibition of human insulin gene transcription by the immunosuppressive drugs cyclosporin A and tacrolimus in primary, mature islets of transgenic mice” Mol. Pharmacol. 63: 1289-1295.

219. Nielsen, J.H.; Mandrup-Poulsen, T.; Nerup, J. (1986) “Direct effects of cyclosporin A on human pancreatic beta-cells” Diabetes 35: 1049-52.

220. Ozbay, L.A.; Smidt, K. (2011) “Cyclosporin and tacrolimus impair insulin secretion and transcriptional regulation in INS-1E beta-cells” Br. J. Pharmacol. 62: 136-46.

221. Kutkuhn, B.; et al. (1997) “Development of insulin resistance and elevated blood pressure during therapy with cyclosporine A” Blood Press 6:13-17.

222. Gillard, P.; et al. (2009) “Functional beta-cell mass and insulin sensitivity is decreased in insulin-independent pancreas-kidney recipients” Transplantation 87: 402-407.

223. Øzbay, L.A.; et al. (2012) “Calcineurin inhibitors acutely improve insulin sensitivity without affecting insulin secretion in healthy human volunteers” Br. J. Clin. Pharmacol. 73(4): 536-545.

224. Whiting, P.H.; et al. (1991) “Toxicity of rapamycin–a comparative and combination study with cyclosporine at immunotherapeutic dosage in the rat” Transplantation 52: 203-208

225. Kneteman, N.M.; Lakey, J.R.; Wagner, T.; Finegood, D. (1996) “The metabolic impact of rapamycin (sirolimus) in chronic canine islet graft recipients” Transplantation 61: 1206-1210.

226. Bell, E.; et al. (2003) “Rapamycin has a deleterious effect on MIN-6 cells and rat and human islets” Diabetes 52: 2731-2739.

227. Fraenkel, M.; et al. (2008). “mTOR inhibition by rapamycin prevents beta-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes” Diabetes 57: 945-957.

228. Cybulski, N.; et al. (2009) “mTOR complex 2 in adipose tissue negatively controls whole-body growth” Proc. Natl. Acad. Sci. USA. 106: 9902-9907.

229. Ichimaru, N.; et al. (2001) “Changes in lipid metabolism and effect of simvastatin in renal transplant recipients induced by cyclosporine or tacrolimus” Atherosclerosis 158: 417-23.

230. Spinelli, G.A.; et al. (2011) “Lipid profile changes during the first year after kidney transplantation: risk factors and influence of the immunosuppressive drug regimen” Transplant Proc. 43: 3730-3737.

231. Chakrabarti, P.; et al. (2010) “Mammalian target of rapamycin complex 1 suppresses lipolysis, stimulates lipogenesis, and promotes fat storage” Diabetes 59: 775-781.

232. Kumar, A.; et al. (2010) “Fat cell-specific ablation of rictor in mice impairs insulin-regulated fat cell and whole-body glucose and lipid metabolism” Diabetes 59: 1397-1406.

233. Wu, J.; Zhu, Y.H.; Patel, S.B. (1999) “Cyclosporin-induced dyslipoproteinemia is associated with selective activation of SREBP-2” Am. J. Physiol. Endocrinol. Metab. 277: E1087-E1094.

234. Ballantyne, C.M. et al. (1989) “Effects of cyclosporine therapy on plasma lipoprotein levels” JAMA 262(1): 53-56.

235. Aguilar-Salinas, C.A.; et al. (2002) “Genetic factors play an important role of pathgenesis of hyperlipidemia post-transplantation” Am. J. Kidney Dis. 40(1): 169-77.

236. Kim, J.E.; Chen, J. (2004) “Regulation of peroxisome proliferator-activated receptor-c activity by mammalian target of rapamycin and amino acids in adipogenesis” Diabetes 53(11): 2748-2756.

237. Asberg, A.; et al. (2009) “Calcineurin inhibitor effects on glucose metabolism and endothelial function following renal transplantation” Clin. Transplant 23: 511-518.

238. Ikeuchi. M.; et al. (1992) “In vivo and in vitro effects of cyclosporin A on glucose transport by soleus muscles of mice” Biochem. Pharmacol. 43: 1459-1463.

239. Øzbay, L.A.; et al. (2012) “Calcineurin inhibitors acutely improve insulin sensitivity without affecting insulin secretion in healthy human volunteers” Br. J. Clin. Pharmacol. 73(4): 536-545.

240. Um, S.H.; et al. (2004) “Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity” Nature 431(7007): 200.

241. Tremblay, F.; et al. (2005). “Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes” Endocrinology 146: 1328-1337.

242. Pereira, M.;et al “Cyclosporine A and tacrolimus reduce cell-surface amount of GLUT4 via increased endocytosis: a potential mechanism for the diabetogenic effects of immunosuppressive agents” (under revision).

243. Taha, C.; et al. (1995) “The insulin-dependent biosynthesis of GLUT1 and GLUT3 glucose transporters in L6 muscle cells is mediated by distinct pathways. Roles of p21ras

244. Wood, I.S.; Trayhurn, P. (2003) “Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins” Br. J. Nutr. 89: 3-9.

245. Carolyn, L.; et al. (2008) “A GSK-3/TSC2/mTOR pathway regulates glucose uptake and GLUT1 glucose transporter expression” Am. J. Physiol. Cell Physiol. 295: C836–C843.

246. Bastard, J.P. et al. (2006) “Recent advances in the relationship between obesity, inflammation, and insulin resistance” Eur. Cytokine Netw. 17: 4-12.

247. Ryden, M.; et al (2004) “Targets for TNF-alpha-induced lipolysis in human adipocytes” Biochem. Biophys Res Commun 318: 168-175

248. Wedellova, Z., Dietrich, et al. (2011). “Adiponectin inhibits spontaneous and catecholamine-induced lipolysis in human adipocytes of non-obese subjects through AMPK-dependent mechanisms”. Physiol Res. 60: 139-48.

249. Pereira, M.; et al. “Effects of immunosuppressive agents on lipid metabolism and gene expression in human adipocytes” (under revision).

250. Jorgensen, P.F.; et al. (2001) “Sirolimus interferes with the innate response to bacterial products in human whole blood by attenuation of IL-10 production” Scand. J. Immunol. 53: 184-189.

251. Schaeffer, V.; et al. (2011) “Role of the mTOR Pathway in LPS-Activated Monocytes: Influence of Hypertonic Saline” J. Surg. Res. 171:769-776.

252. Neal, J.W.; Clipstone, N.A.; (2002) “Calcineurin mediates the calcium-dependent inhibition of adipocyte differentiation in 3T3-L1 cells” J. Biol. Chem. 277(51):

253. Smith, S.J.; et al. (2000). "Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat". Nat. Genet. 25 (1): 87–90.

254. Liang H; Ward WF (2006) “PGC-1: a key regulator of energy metabolism” Adv Physiol Educ 30: 145-151

255. Yoon, J.C. (2001) “Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1” Nature 413: 131–138.

256. Patti, M.E.; et al. (2003) “Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1” Proc. Natl. Acad. Sci. USA 100: 8466-8471.

257. Kitamura, T.;,et al. (2007) “A Foxo/ Notch pathway controls myogenic differentiation and fi ber type specifi cation” J. Clin. Invest. 117: 2477–2485.

258. Hribal, M.L.; et al. (2003) “Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors” J. Cell Biol. 162: 535-541.

259. Kamei, Y.; et al. (2004) “Skeletal muscle FOXO1 (FKHR) transgenic mice have less skeletal muscle mass, down-regulated Type I (slow twitch/red muscle) fiber genes, and impaired glycemic control” J. Biol. Chem. 279: 41114–41123.

260. Southgate, R.J.; et al. (2007) “FOXO1 regulates the expression of 4E-BP1 and inhibits mTOR signaling in mammalian skeletal muscle” J. Biol. Chem. 282: 21176–21186.

261. Allen, D.L.; Unterman, T.G. (2007) “Regulation of myostatin expression and myoblast differentiation by FoxO and SMAD transcription factors” Am. J. Physiol. Cell Physiol. 292: C188–C199

262. Wu, A.L.; et al. (2008) “Forkhead box protein O1 negatively regulates skeletal myocyte differentiation through degradation of mammalian target of rapamycin pathway components” Endocrinology 149: 1407–1414.

263. Nakae, J.; et al.; (2003) “The forkhead transcription factor Foxo1 regulates adipocyte differentiation” Dev. Cell 4: 119–129.

264. Qu, S.; et al. (2006) “Aberrant Forkhead box O1 function is associated with impaired hepatic metabolism” Endocrinology 147:25641–5652.

265. Matsumoto, M.; Han, S.; Kitamura, T.; Accili, D. (2006) “Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism” J. Clin. Invest. 116: 2464–2472.

266. Kamagate, A.; et al. (2008) “FoxO1 mediates insulin-dependent regulation of hepatic VLDL production in mice” J. Clin. Invest. 118: 2347–2364.

267. Foretz, M.; et al. (1999). “ADD1/SREBP-1c is required in the activation of hepatic lipogenic gene expression by glucose”. Mol. Cell Biol. 19: 3760-3768.

268. Iizuka, K.; Horikawa, Y. (2008) “ChREBP: a glucose-activated transcription factor involved in the development of metabolic syndrome” Endocr J. 55(4): 617-24.

269. Ruderman, N.B.; Saha, A.K.; Kraegen, E.W. (2003) “Malonyl CoA, AMP-activated protein kinase, and adiposity” Endocrinology 144: 5166–5171.

270. Roden, M., et al. (1996). “Mechanism of free fatty acid-induced insulin resistance in humans”. J Clin Invest. 97: 2859-65.

271. Baba, H.; Zhang, X.J.; Wolfe, R.R. (1995) “Glycerol gluconeogenesis in fasting humans. Nutrition”. 11: 149-53.