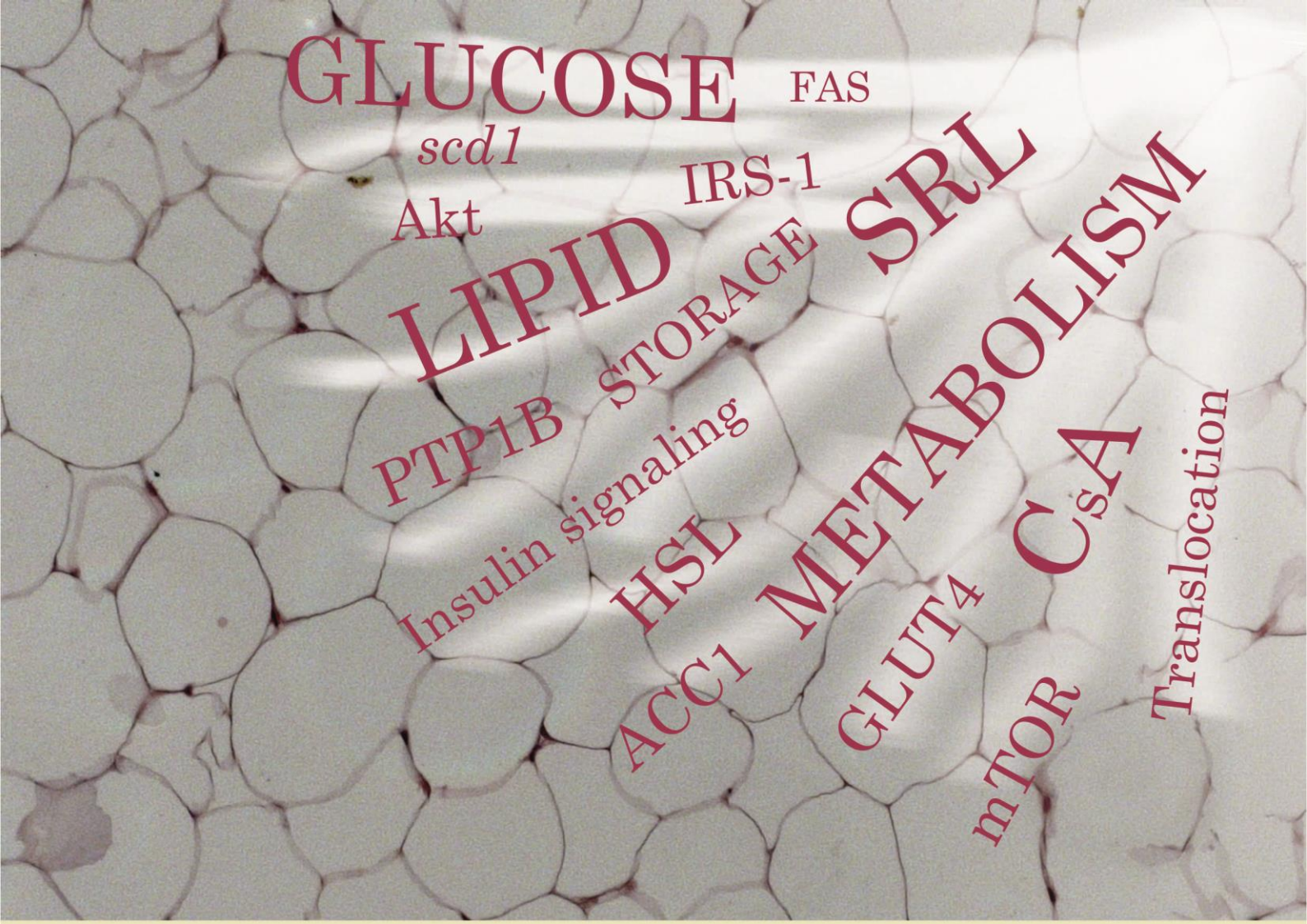

Patrícia Cristina Moura Martins Lopes

MOLECULAR MECHANISMS INVOLVED IN GLUCOSE AND LIPID METABOLISM AFTER IMMUNOSUPPRESSIVE THERAPY

Doctoral Thesis in Biosciences, specialization in Biochemistry, supervised by Doctor Eugénia Carvalho, co-supervised by Professor Doctor Carlos Palmeira and presented to the Life Sciences Department of the Faculty of Sciences and Technology of the University of Coimbra.

2014

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UNIVERSIDADE DE COIMBRA

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Cover: Image of adipocytes stained with hematoxylin/eosin. Illustration created by Hugo Alves.

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Mecanismos moleculares envolvidos no metabolismo da glucose e dos lípidos após terapia imunossupressora

Tese de Doutoramento em Biociências, especialização em Bioquímica, orientada pela Doutora Eugénia Carvalho e co-orientada pelo Professor Doutor Carlos Palmeira e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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This work was performed at the Center for Neuroscienece and Cell Biology (CNBC), University of Coimbra, under the supervision of Doctor Eugénia Carvalho (CNBC, University of Coimbra) and co-supervision of Professor Doctor Carlos Palmeira (CNBC and Department of Life Sciences, University of Coimbra). Its execution was supported by a PhD fellowship from the Portuguese Foundation for Science and Technology (SFHR/BD/61405/2009).

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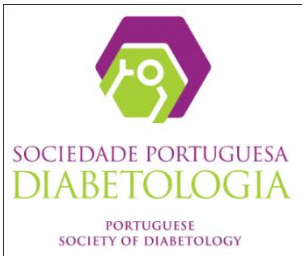

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List of acronyms and abbreviations

ACC	Acetyl-CoA carboxylase
Akt	Protein kinase B
Apo	Alipoprotein
AS160	Protein kinase B substrate of 160 kDa
ATGL	Adipose triacylglycerol lipase
ChREBP	Carbohydrate-responsive element-binding protein
CNI	Calcineurin inhibitor
CPN	Cyclophilin
Ct	Threshold cycle
CsA	Cyclosporine A
DG	Diglyceride
DGAT	Diglyceride acyltransferase
DNL	*De novo* lipogenesis
ELISA	Enzyme –Linked Immuno-Sorbent-Assay
FKBP12	FK506-binding protein (12-kD)
FAS	Fatty acid synthase
FA	Fatty acid
FFA	Free Fatty acid
FOX	Forkhead box
GK	Glucokinase
GLUT	Glucose transporter
GTT	Glucose tolerance test
G6P	Glucose-6 phosphate
G6Pase	Glucose-6-phosphatase
HDL	High-density lipoprotein
HSL	Hormone-sensitive lipase
IA	Immunosuppressive agent
IL-6	Interleukin-6
IR	Insulin receptor
IRS	Insulin receptor substrate
ITT	Insulin tolerance test

LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MG	Monoacylglycerol
NEFA	Non-esterified fatty acid
NFAT	Nuclear factor of activated T cells
NODAT	New onset diabetes after transplantation
NMR	Nuclear magnetic ressonance
p70S6K	p70 ribosomal S6 kinase
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase 1
PEPCK	Phosphoenolpyruvate carboykinase
PI3K	Phosphatidylinositol 3-kinase
PGC1-α	Peroxisome proliferator-activated receptor- coactivator
PKA	Protein kinase A
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor γ
PTP1B	Protein-tyrosine phosphatase 1B
SREBP	Sterol regulatory element-binding proteins
SRL	Sirolimus
Tac	Tacrolimus
TG	Triglyceride
TNF-α	Tumor necrosis factor - α
VLDL	Very low density lipoprotein
WAT	White adipose tissue

Abstract

Diabetes mellitus is a widespread and growing public health problem due mainly to the aging of the population and changes in diet and life style. The clinical diagnosis of diabetes has increased worldwide, including in Portugal. About one third of the population is pre-diabetic and/or undiagnosed diabetic. Diabetes is associated with the metabolic syndrome, which is characterized by several risk factors, including insulin resistance and dyslipidemia. These are two of the main metabolic complications that may also appear after transplantation. Organ transplant is a therapeutic measure of last resort for patients with end-stage diseases who have exhausted all other available treatments without improvement. The most important issue in organ transplantation is to ensure the graft versus host survival; in the last decades, advances in immunosuppressive therapy have led to an important improvement. Calcineurin inhibitors, such as cyclosporine A (CsA), are cornerstones of immunosuppressive therapy; however recently other agents, like sirolimus (SRL), a mammalian target of rapamycin (mTOR) inhibitor, have been developed in order to produce protocols that able to minimize the use of calcineurin inhibitors. Although very effective in their functions as immunossupressors, both agents are associated with new onset diabetes after transplantation (NODAT) and dyslipidemia. Development of these metabolic complications increases the risk for graft failure and patient death. However, the molecular mechanisms underlying these metabolic effects are not fully elucidated and animal's studies are important to clarify these aspects. In this thesis we aimed to understand the effects of therapeutic doses of CsA (5 mg/kg/day) and SRL (1 mg/Kg/day) in glucose and lipid metabolism in peripheral insulin sensitive tissues, such as adipose tissue, muscle and liver in an *in vivo* rat model, after short and long treatments.

The CsA-treated group presented an impaired response to glucose during a glucose tolerance test, particularly at the 15 min time point. The glucose excursion curve for SRL was also impaired, as the recovery kinetics of blood glucose levels were slower, compared to the vehicle group. Furthermore, during an ITT, the decrease in blood glucose levels in the CsA-treated group was delayed 60 min, compared to the vehicle group. Interestingly, after 9 weeks, SRL-treated animals were hyperinsulinemic while CsA-treated animals presented lower insulin values, suggesting glucose intolerance and insulin resistance in both treated groups.

Moreover, a significant reduction in the insulin-stimulated glucose uptake over basal was observed in isolated adipocytes, whether treated *ex vivo* or *in vivo* with CsA and SRL. Phosphorylation of the main proteins in the insulin cascade, namely IR (insulin receptor) at Tyr1146, IRS-1 (insulin receptor substrate-1) at Tyr612, Akt/PKB (protein kinase B) at both Ser473 and Thr308 was suppressed in the SRL group, which could explain the observed reduction in glucose uptake. In fact, impaired Akt/PKB activation leads to a decrease in phosphorylation of the substrate, AS160, which might block the insulin-stimulated translocation of glucose transporters to the cell membrane and therefore, the glucose uptake. Although none of the proteins involved in the insulin cascade were significantly affected by the CsA treatment, effects on key enzymes for hepatic gluconeogenesis suggest that CsA stimulates this mechanism. In fact, CsA increased protein levels of two of the enzymes involved in gluconeogenesis, glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) in the liver as well as the transcription factors peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) and forkhead box protein O1 (FOXO1), which may contribute to enhanced gluconeogenesis. We also observed a significant increase in protein-tyrosine phosphatase 1B (PTP1B), a negative regulator of insulin, in the CsA-treated group in the liver, indicating increased insulin resistance.

CsA-treated animals presented an increase in serum non-esterified fatty acid (NEFA) and triglycerides (TGs) after 9 weeks of treatment. Moreover, CsA and SRL treatments of rats for 3 and 9 weeks increased isoproterenol-stimulated lipolysis in isolated adipocytes by 5-9 fold and 4-6 fold, respectively. The increase in lipolysis might have been due to the observed increase in the expression of the main lipolytic protein, hormone-sensitive lipase (HSL). SRL treatment also caused ectopic deposition of TGs in liver and muscle after 3 weeks. Additionally, SRL treatment reduced the expression of lipogenic genes, including acetyl-CoA carboxylase 1 (ACC1), lipin 1, peroxisome proliferator-activated receptor gamma (PPAR-γ) and stearoyl-CoA desaturase (SCD1) in adipose tissue. The reduced expression of lipogenic factors in adipose tissue might have impaired lipid storage on this tissue, and contributed to the observed ectopic deposition of fat in liver and muscle.

Furthermore, we used a higher dose of CsA (15mg/kg/day) *in vivo* for 15 days, in order to evaluate CsA effects in glucose and lipid metabolism. This was done through quantification of ^{2}H-enrichment of glucose, glycogen and TG after $^{2}H_2O$ administration by ^{2}H NMR. Although we determine that CsA at this dose affects body weight and glucose

tolerance, we could not see differences in glycogen synthesis or *de novo* lipogenesis, under these conditions.

In conclusion, the molecular and metabolic changes observed in this work contributes to a better understanding of the mechanisms involved in the development of NODAT and dyslipidemia after immunosuppressive therapy, thus opening new possibilities for prevent these serious side-effects and improve grafts and patients survival.

Resumo

Diabetes mellitus é uma doença que tem vindo a aumentar em todo o Mundo, devido ao envelhecimento da população e à alteração do estilo de vida, tendo-se tornado num problema de saúde pública. Um terço da população encontra-se num estado de pré-diabetes ou diabetes não diagnosticada. A diabetes está associada à síndrome metabólica, que é caracterizada por diversos fatores de risco, nomeadamente resistência à insulina e dislipidemia. Estas duas complicações metabólicas podem ocorrer após um transplante de órgãos, que é o último recurso terapêutico para pacientes com doença em estádio terminal. A questão mais importante é garantir a prevenção da rejeição do transplante pelo hospedeiro; recentes avanços na terapia imunossupressora têm vindo a aumentar a taxa de sucesso. Os inibidores de calcineurina, como a ciclosporina A (CsA), são um marco terapêutico, no entanto, nos últimos anos outros fármacos como o Sirolimus (SRL), um inibidor do alvo da rapamicina nos mamíferos (mTOR), foram desenvolvidos. De facto, apesar de muito eficazes, estes dois fármacos estão associados a efeitos secundários, incluindo diabetes pós-transplante e dislipidemia, que aumentam o risco de rejeição do transplante e morte de paciente. Como os mecanismos moleculares subjacentes a estes efeitos metabólicos não estão totalmente elucidados, é da maior importância utilizar modelos animais para os estudar *in vivo*. O principal objetivo deste estudo foi avaliar, em ratos Wistar, os efeitos de doses terapêuticas de CsA (5 mg/kg peso/dia) e SRL (1 mg/Kg peso/dia) administradas de forma aguda (3 semanas) e crónica (9 semanas) no metabolismo da glucose e de lípidos, nos tecidos periféricos sensíveis à insulina – fígado, músculo e tecido adiposo.

Os animais tratados com CsA apresentaram uma resposta insuficiente no teste de tolerância à glucose, uma vez que a concentração de glucose era mais elevada 15 minutos depois de a glucose ser administrada comparativamente ao grupo veículo. Nos animais tratados com SRL, verificou-se também alteração da curva de concentração de glucose, pois a cinética de recuperação da concentração de glucose no sangue foi mais lenta. Durante o teste de tolerância à insulina em animais tratados com CsA, a concentração de glucose diminuiu ao fim de 60 minutos, requerendo mais tempo que os animais do grupo veículo. Após 9 semanas de tratamento os animais tratados com SRL estavam hiperinsulinémicos, enquanto os animais tratados com CsA apresentavam concentrações mais baixas de insulina, comparativamente aos animais do grupo veículo, sugerindo intolerância à glucose e resistência à insulina em ambos os grupos.

Verificou-se uma redução significativa na captação da glucose por adipócitos isolados estimulados por insulina, em comparação com o nível basal, quer para os adipócitos tratados *ex vivo* com CsA e SRL, quer para aqueles provenientes dos estudos *in vivo*. Também, se observou, nos animais tratados com SRL, a inibição da fosforilação das principais proteínas da via de sinalização de insulina, nomeadamente no resíduo Tyr1146 do recetor de insulina (IR), no resíduo Tyr612 do substrato do recetor de insulina (IRS-1) e dos resíduos Ser473 e Thr308 da proteína quinase B (Akt), o que pode explicar a redução observada na captação da glucose. De facto, a diminuição da ativação da Akt conduz à diminuição na fosforilação do substrato AS160, o que pode levar ao bloqueio da translocação dos transportadores de glucose para a membrana plasmática das células e consequentemente a captação da glucose. Em contraste, o tratamento com CsA não afetou a fosforilação de nenhuma das proteínas desta via de sinalização. No entanto, o tratamento com CsA induziu a gliconeogénese dado o aumento da expressão proteica de duas enzimas envolvidas neste processo – G6Pase e PEPCK, assim como dos fatores de transcrição PGC1-α e FOXO1. Também se observou, no fígado dos animais tratados com CsA, um aumento do PTP1B, regulador negativo da insulina, indicando resistência de insulina.

Os animais tratados com CsA apresentaram, após 9 semanas de tratamento, um aumento da concentração de NEFA e TGs em circulação. Verificou-se também o aumento significativo da lipólise em adipócitos isolados, estimulados com isoproterenol, em ambas as durações do tratamento e com ambos os fármacos, quando comparada com a lipólise em adipócitos do grupo veículo. A indução da lipólise pode dever-se ao aumento observado na expressão de uma das mais importantes proteínas lipolíticas, a HSL. Estes resultados sugerem que o aumento da lipolise *in vivo* com CsA e o SRL pode contribuir para a dislipidemia observada durante a terapia imunossupressora. O tratamento com SRL durante 3 semanas causou deposição ectópica de TG no músculo e fígado e reduziu a expressão de genes lipogénicos no tecido adiposo, nomeadamente ACC1, lipin-1, PPAR-γ e SCD1. Esta redução pode levar a uma diminuição no armazenamento da gordura no tecido adiposo e contribuir para a deposição de gordura observada no fígado e músculo.

Para avaliar os efeitos no metabolismo da glucose e lípidos através da identificação de metabolitos por ^{2}H NMR foi administrada uma dose mais elevada de CsA (15mg/kg peso/dia) durante 2 semanas. Foi feita a determinação da glucose, do glicogénio e dos TG, que incorporaram ^{2}H proveniente de água deuterada ($^{2}H_2O$). Embora tenha sido observado que nesta dose a CsA diminui o peso dos animais e a tolerância à glucose, não foram detetadas diferenças significativas na síntese de glicogénio nem na lipogénese *de novo*.

Em conclusão, as alterações metabólicas encontradas neste trabalho podem ajudar a revelar a origem do desenvolvimento da diabetes pós-transplante e dislipidemia depois da terapia imunossupressora, abrindo assim novas possibilidades de prevenção destes efeitos secundários, como forma de melhorar a sobrevivência dos enxertos e dos doentes transplantados.

List of publications

The thesis is based on the following papers

1.**Lopes, PC**, Fuhrmann, A, Sereno, J, Pereira MJ, Nunes, P, Pedro, JR, Melão, A, Reis, F. Carvalho, E. (2013) Effects of Cyclosporine and Sirolimus on Insulin-Stimulated Glucose Transport and glucose Tolerance in a Rat Model. *Transplantation Proceedings*. 45 (3) 1142-8.

2. **Lopes, PC**, Fuhrmann, A, Sereno, J, Pereira MJ, Eriksson, JW, Reis, F. Carvalho, E. Cyclosporine A enhances gluconeogenesis while sirolimus impairs insulin signaling in peripheral tissues after 3 weeks of treatment (*submitted to Biochemical Pharmacology*).

3. **Lopes, PC**, Fuhrmann, A, Sereno, J, Pereira MJ, Eriksson, JW, Reis, F. Carvalho, E. Short and long-term effects of Cyclosporine A and Sirolimus on genes and proteins involved in lipid metabolism *in vivo* in Wistar rats (*Metabolism, Clinical and Experimental, in press*).

4. **Lopes PC**, Jarak I, Jones JG, Carvalho E.
NMR-based metabolic profiling of hepatic responses to Cyclosporin A *(in preparation).*

CHAPTER 1

Introduction

1.1 *Diabetes mellitus* and its metabolic complications

Diabetes mellitus is a widespread and growing public health problem due mainly to the aging of the population and changes in the life style (Shaw *et al.*, 2010). Diabetes is associated with the metabolic syndrome, which is characterized by several risk factors, such as insulin resistance, abdominal obesity, atherogenic dyslipidemia, hypertension, hyperuricemia, a prothrombotic state, and a pro-inflammatory state (Grundy *et al.*, 2004; Petersen *et al.*, 2007 ; Reaven, 1988). Clinical diagnosis of diabetes has been increasing worldwide and currently, about 150 million people suffer from type 2 diabetes; the prediction is that by 2030 over 439 million people worldwide will suffer from type 2 diabetes with a severe socioeconomic impact (Shaw *et al.*, 2010). In fact, about 16 to 17 million people are pre-diabetic, having early symptoms but not yet the full manifestation of the disease. In Portugal, about one third of the population (34.9%) aged 20-70 years is pre-diabetic and a high percentage of the population is undiagnosed (43.6%) (Gardete-Correia *et al.*, 2010). Genetic predisposition is also important to the development of type 2 diabetes. Healthy individuals, who have two first-degree relatives with *Diabetes mellitus,* have a three-to five-fold greater risk of developing the disease than those without a family history of diabetes (Carvalho *et al.*, 1999; Elbein *et al.*, 1991). In developed countries the prevalence of diabetes is higher than in the rest of the world, however, in near future, the major increase in people with diabetes will occur in developing countries (Badaru *et al.*, 2012), mostly due

to the improvement in living conditions, longer life expectancy and changes in life style (Figure 1.1).

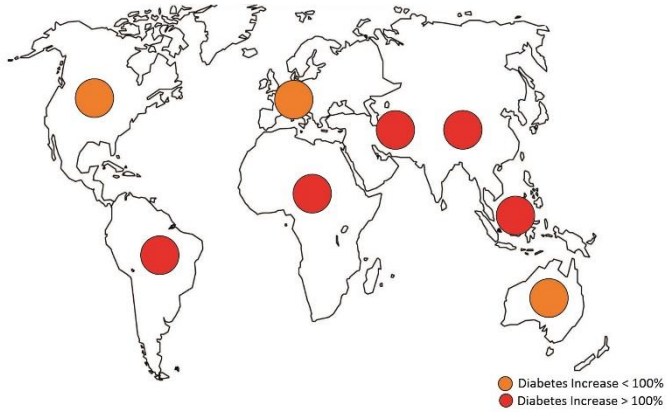

Figure 1.1 - Diabetes increase worldwide.
The major increase in people with diabetes will occur in developing countries, namely South America, Africa, India, China and Southern Asia.

Etiologically, diabetes is a disease that can be divided in four main types: type 1, type 2, gestational diabetes and secondary diabetes. Type 1 diabetes, which usually appears in childhood and represent about 10% of all cases of diabetes, is caused by autoimmune destruction of pancreatic β-cells with consequent deficiency in insulin production (ADA, 2004). On the other hand, type 2 diabetes is more common and is considered a heterogeneous disease, caused by multiple factors and the result of peripheral insulin resistance and/or a β-cell secretory defect. Gestational diabetes is diagnosed after glucose intolerance is identified during pregnancy and usually reverses after delivery. The last type – secondary diabetes, which is our focus, can be caused by genetic defects that affect β-cells function (like maturity onset diabetes), diseases of exocrine pancreas, endocrinopathies and pancreatic dysfunction and/ or insulin resistance caused by drugs, like immunosuppressive agents (ADA, 2004).

In fact, insulin resistance or diabetes is one of the metabolic complications that may appear after transplantation. Organ transplant is the medical procedure used when all the other treatments are exhausted, and is applied at the end stage of organ failure. In the USA alone, 28.051 people received organ transplants, while in Portugal 681 people were transplanted, in 2012 (Instituto Português de Sangue e Transplantação; organdonor.gov).

The one-year graft survival rates for kidney, liver and heart transplantations are reported between 82% and 95% (Burket *et al.*, 2008). However, in order to totally accomplish a successful transplant, allograft rejection must be prevented and for this purpose immunosuppressive therapy is essential.

1.2 Immunosuppressive agents

Over the past decades, advances in immunosuppressive therapy led to an important improvement in graft survival. The constant research and development of new pharmaceutical agents, allows the transplant community to create regimens that can improve graft survival rates. This success is linked to the reduction of side effects, including new onset diabetes after transplantation (NODAT), dyslipidemia, cardiovascular disease, nephrotoxicity, malignancy and cosmetic effects, such as acne and gingival hyperplasia (Da Silva *et al.*, 2012; Momin *et al.*, 2010; Smith *et al.*, 2003).

Protocols for immunosuppression normally include different agents in order to achieve the maximum efficiency in preventing organ rejection. Glucocorticoids, which already exist naturally in our body as endogenous cortisol, are commonly used in immunosuppression protocols, in the form of exogenous therapeutic agents such as prednisone, methylprednisolone and dexamethasone. These agents have been used in the clinic since 1920s and was one of the first classes of medications used to prevent rejection after solid organ transplantation. In fact, glucocorticoids are very successful on interrupting several steps in immune activation, inhibiting cytokine production, proliferation of lymphocytes and promoting changes in cell trafficking (Baxter, 1992; Steiner *et al.*, 2011). Another class of agent is calcineurin inhibitors (CNI), which includes cyclosporine A (CsA) and tacrolimus (Tac), also cornerstones of immunosuppressive therapy. More recently, other immunosuppressive agents (IA) have become available such as anti-proliferative agents like mycophenolate mofetil and inhibitors of mammalian target of rapamycin (mTOR), like sirolimus (SRL, also known as rapamycin) and everolimus (Smith *et al.*, 2003). These new agents allowed the development of new protocols in order to minimize the usage of calcineurin inhibitors or glucocorticoids, which presented severe side effects in spite of their good outcome on preventing allograft rejection. Although these protocols are center-specific, a higher immunosuppressive load is commonly used at an early phase of the transplant (induction phase). The induction phase includes an antibody therapy in combination with calcineurin inhibitors, glucocorticoids and an anti-proliferative agent (Scherer *et al.*, 2007). On the other hand, in the maintenance phase, protocols with CNI,

glucocorticoids minimization or withdrawal and conversion to mTOR inhibitor and/or other anti-proliferative agents are applied. These protocols are dependent of the type of transplanted organ and pre-existing conditions in the patient, and focused on "individual tailored immunosuppressive protocol" (Beckebaum *et al.*, 2013; Scherer *et al.*, 2007).

The use of CsA and SRL is mentioned with more detail in the next section, since they are the focus of this thesis.

1.2.1 Calcineurin inhibitors

Calcineurin is a Ca^{2+}/calmodulin dependent serine/threonine phosphatase (Klee *et al.*, 1979; Klee *et al.*, 1998; Stewart *et al.*, 1982). It is present in different tissues and is responsible for different cellular functions, that include control of intracellular Ca^{2+} signaling, gene regulation and external signal-mediated biological responses (Crabtree, 1999; Stankunas *et al.*, 1999). It is also responsible for the regulation of transcription of the T-cell growth factor, interleukin-2 (Schreiber *et al.*, 1992). The dephosphorylation of the transcription factor NF-AT (nuclear factor of activated T cells) is required for its translocation from the cytoplasm to the nucleus, in response to an increased intracellular Ca^{2+} level (Bandyopadhyay *et al.*, 2002). In order to suppress the immune system a particular class of drugs that inhibit calcineurin and the translocation of NF-AT have been developed, which includes CsA.

CsA was discovered at the Sandoz Laboratories (now Novartis) in the 1970s. It is a cyclic peptide of fungal origin *(Tolypocladium inflatum)* that binds to cyclophilins (a family of cellular proteins), to form a complex that inhibits calcineurin. Consequently, NF-AT dephosphorylation and its translocation into the nucleus are prevented, blocking interleukin-2 gene expression, decreasing proliferation and differentiation of T-cells and overall the suppression of the immune response (Sarwal *et al.*, 2001; Smith *et al.*, 2003) (Figure 1.2).

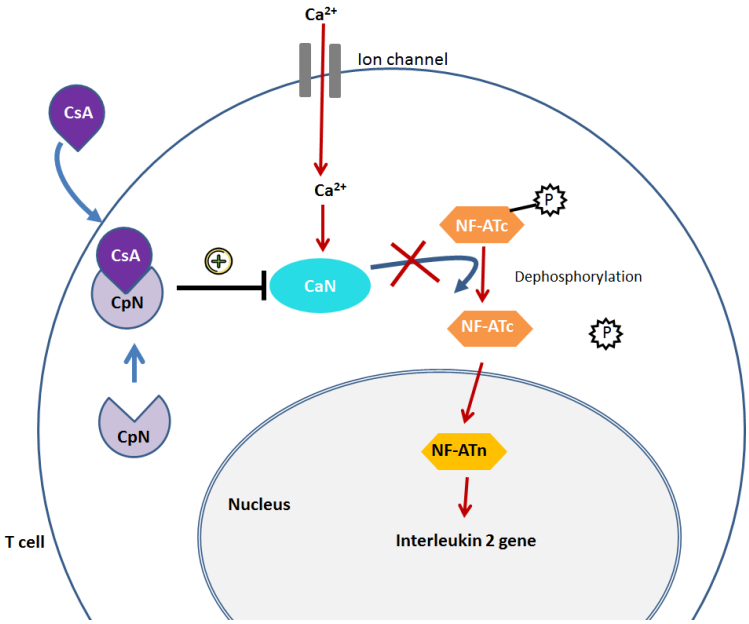

Figure 1.2. Mechanism of action of CsA for the inhibition of the immune system. Cyclosporine A (CsA); Ciclophilin (CpN); calcineurin (CaN); nuclear factor of activated T cells (NF-AT); calcium (Ca^{2+})

As a suppressor of the immune system, CsA was approved by the US Food and Drugs Administration (FDA) in 1983, to be used in the prevention of organ rejection in kidney, liver and heart transplant recipients (Lindenfeld *et al.*, 2004). The introduction of CsA led to improvement and success in the transplantation field (Heusler *et al.*, 2001). The clinical recommended oral doses for CsA in the early post-transplant phase is between 12 to 15 mg/kg/day and generally, steady state trough concentrations are measured every day for the first 2 weeks after transplantation or until the patient is stable. Thereafter, concentrations are measured once a week for the first month and then monthly for the first 12 months after transplantation and the maintenance dose varies from 4 to 8 mg/kg/day (Harmon *et al.*, 1993; Lindenfeld *et al.*, 2004; Smith *et al.*, 2003).

Although, very successful in its function, CsA is associated with several side effects, including nephrotoxicity, hypertension, dyslipidemia and NODAT (Subramanian *et al.*, 2007). Nonetheless, it is still not clear if the relative diabetogenicity of calcineurin inhibitors is due to β-cells dysfunction, systemic insulin resistance or both.

1.2.2 – mTOR inhibitors

The mammalian target of rapamycin (mTOR) is a kinase that integrates inputs from many nutrients, growth factors and insulin. It transmits the signal to downstream targets to adjust cell growth and proliferation as well as metabolic homeostasis (Hay *et al.*, 2004).

mTOR exists as two physically and functionally distinct multiprotein complexes located in the cytoplasm, termed the mTOR complex 1 (mTORC1, mTOR-raptor) and the mTOR complex 2 (mTORC2, mTOR-rictor) (Dowling *et al.*, 2010; Rosner *et al.*, 2008). Functionally, mTORC1 is sensitive to nutrients, especially amino acids, whereas mTORC2 regulates cell proliferation and survival, as well as cytoskeletal reorganization (Madke, 2013).

SRL (or rapamycin) is an antifungal macrolide produced by the bacterium *Streptomyces hygroscopicus*, isolated in 1970, and approved by the FDA in 1999 to be used as an immunosuppressive agent (Sehgal *et al.*, 1975). SRL binds to the 12-kD FK506-binding protein (FKBP12) and this complex inhibits the target of rapamycin (TOR) Ser/Thr kinase. As mTOR regulates mRNA translation initiation and progression from the G1 to S phase of the cell cycle, its inhibition will prevent the T-cell proliferation (Chung *et al.*, 1992) (Figure 1.3).

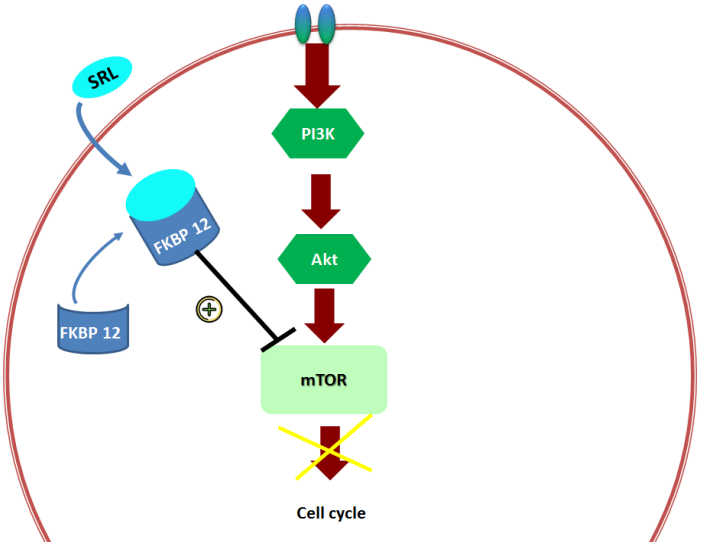

Figure 1.3: Mechanism of action of SRL for the inhibition of the immune system.
Sirolimus - SRL; FK506-binding protein 12kDa (FKBP12); phosphoinositide 3-kinase (PI3K); Protein kinase B (Akt/PKB); mammalian target of mTOR (mTOR)

SRL can be an alternative to calcineurin inhibitors following transplantation, due to its antiproliferative properties and consequently antitumoral or antiatherogenic activity, and higher safety against renal toxicity (Cravedi P, 2010; Mehrabi A, 2006). It can be used alone, or in conjunction with other agents namely calcineurin inhibitors and/or mycophenolate mofetil, to provide steroid-free immunosuppression regimens. SRL is primarily used in renal transplantation and patients are given an initial loading dose of 12 to 20 mg/kg/day, followed

by a reduction of 4 to 8 mg /kg/day until day 5-7, and then the dose is adjusted in order to achieve steady-state circulatory trough levels of approximately 8 to 20 ng/ml in the maintenance phase. The sirolimus oral solution is normally administered once daily in the morning after dilution with water or orange juice (Schena *et al.*, 2009).

Although most of the time, SRL is chosen for renal transplants as it is associated to a low rate of nephrotoxicity, the use of this immunosuppressive agent is not consensual between transplant centers as to the right moment to use it and the duration of treatment. Its use has been associated with an increased risk of acute rejections and worse graft function as compared with CsA or Tac (Cravedi *et al.*, 2010; Ekberg *et al.*, 2007).

1.3 Long-term metabolic complications due to immunosuppressive agents

As already mentioned, one of the most concerning side effects related to the use of immunosuppressive agents (IAs) after transplantation is the development of metabolic complications, including an impairment of glucose tolerance and insulin secretion, as well as an increase in circulating lipids, leading to a diagnosis of diabetes. As an increasing problem in public health, several medications have been developed to control many aspects of diabetes. Nonetheless, macrovascular and microvascular complications continue to be manifested, affecting not only the vascular system, but also the kidney and peripheral nerves, with severe consequences, that affect the longevity and quality of life of patients that have been submitted to organ transplantation (ADA, 2004).

1.3.1 -New-onset diabetes after transplantation (NODAT)

The main characteristics of NODAT are similar to type 2 diabetes; i.e. decreased insulin secretion and peripheral insulin resistance (Hagen M, 2003; Hur *et al.*, 2007). Hyperglycemia occurs due to the imbalance between insulin production by the β-cells and the target tissue insulin demand (Chow *et al.*, 2008). In accordance, the diagnosis of NODAT is based on the American Diabetes Association criteria for the diagnosis of type 2 diabetes (ADA, 2004; Chow *et al.*, 2008; Markell, 2004) as endorsed by the published international consensus guidelines (Davidson *et al.*, 2003). Nonetheless, some factors that lead to this outcome might be present before the transplant. In fact, some patients that eventually developed NODAT had previous glucose intolerance or insulin resistance (Chow *et al.*, 2008). It is believed that the main risk factors for developing NODAT are familial history of diabetes, genetic predisposition, pre-transplant fasting hypertriglyceridemia, obesity, older recipient age at the time of transplant and non-white ethnicity (African Americans and

Hispanics) (Guerra *et al.*, 2012; Luan *et al.*, 2008; Montori *et al.*, 2002; Vincenti *et al.*, 2007). NODAT is usually manifested in the first few months post-transplantation, being the incidence rate variable due to the type of transplant, the target population or the kind of therapy used. According to Montori *et al.* (2002), 74% of the differences encountered in NODAT incidence between the different studies is due to the IA regimen used. Nonetheless, NODAT incidence varies from 15 to 78% for renal transplant patients in the first year, while it is about 45% for liver recipients. NODAT may not necessarily be permanent as it has long been recognized that it may resolve within weeks or months with changes in the regiment of the immunosuppressive agents used or with antidiabetic agents (Chow *et al.*, 2008; Rossetto *et al.*, 2010; Sulanc *et al.*, 2005; Woodward RS, 2003). We also have to take in consideration, that as a result of a better quality of life post-transplant, an increase in caloric intake and weight gain is expected (Luan *et al.*, 2008). However, complete remission from diabetes is difficult to predict as some patients that undergo apparent remission may later on develop persistent hyperglycemia (Sulanc *et al.*, 2005).

Whereas glucocorticoids, CsA and Tac have been the major agents responsible for affecting glucose homeostasis after solid organ transplantation (Subramanian *et al.*, 2007). SRL is mostly associated with dyslipidemia, and to a lesser extent with NODAT, being this matter still a question of debate in the field. In fact, SRL has been associated with higher risk of developing NODAT in large North American and European cohorts and with lesser risk in other centers. The risk is particularly high when SRL is associated with calcineurin inhibitors (Cravedi P, 2010; Flechner *et al.*, 2011; Ghisdal *et al.*, 2012; Johnston *et al.*, 2008; Morrisett *et al.*, 2002; Veroux *et al.*, 2008).

1.3.2 Dyslipidemia

After glucocorticoids, CsA and SRL are the most common cause for the development of post-transplant dyslipidemia, and it appears to be dose and duration of treatment-related (Subramanian *et al.*, 2007), with an incidence of 20 to 80% in the first year (Kesten *et al.*, 1997; Parekh *et al.*, 2012). In liver transplant, hypertriglyceridemia seems to be mainly related with CsA, although this idea is not consensual. Renal transplanted patients under SRL therapy developed dyslipidemia, with an increase in cholesterol and triglycerides (TGs), and more precisely low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and non-High-density lipoprotein cholesterol (non-HDL-C) (Claes *et al.*, 2012; Morrisett *et al.*, 2002). Approximately 60% of patients treated with mTOR inhibitors in clinical trials had to receive lipid-lowering therapy (Kasiske *et al.*, 2008). Moreover,

combination of calcineurin inhibitors with m-TOR inhibitors increases the risk of developing dislipidemia (Anastacio *et al.*, 2010).

The main issue is that NODAT and dyslipidemia are associated with an increased risk of graft failure (60%) or even more, with an increased risk of patient death (90%) (Chow *et al.*, 2008; Kasiske *et al.*, 2003). In fact, elevated glucose levels, and dyslipidemia are conditions associated with an increased risk of cardiovascular disease in transplant recipients and also with decreased rates of allograft and patients survival (Del Castillo *et al.*, 2004).

In order to elucidate the molecular mechanisms behind the development of both NODAT and dyslipidemia after immunosuppressive therapy, the main peripheral tissues must be address. Adipose tissue, muscle and liver seem to be the most critical in this sense, and are the object of the present thesis.

1.4 Primary peripheral tissues affected by immunosuppressive agents

1.4.1 Adipose tissue

Adipose tissue has been viewed for many years as an inert organ, functioning as a TG store. Nonetheless, this notion has changed and the adipocyte has now gained a new "status" among researchers. Adipose tissue play a critical role in energy homeostasis, interacting with neural/sympathetic (e.g. adrenergic) or hormonal stimulus (e.g. insulin), responding rapidly to changes in nutritional levels and also secreting cytokines (e.g. adiponectin and leptin) (Ahima, 2006; Bjorndal *et al.*, 2011). Different types of adipose tissue have been described according to their location and function (Gesta *et al.*, 2007). White adipose tissue (WAT) is the predominant type of adipose tissue in mammals. This tissue is composed of adipocytes, surrounded by loose connective tissue, highly vascularized and innervated, containing various cell types such as macrophages, fibroblasts and adipocyte precursors (Figure 1.4). The capacity for WAT to store triglycerides is believed to be almost unlimited, however expansion of adipose tissue has a limit, that when reached can cause the body to store fat in non-adipose tissues, such as liver, heart, muscle and pancreas, developing a toxic response known as lipotoxicity (Ahima, 2006; Medina-Gomez *et al.*, 2007; Stephens, 2012).

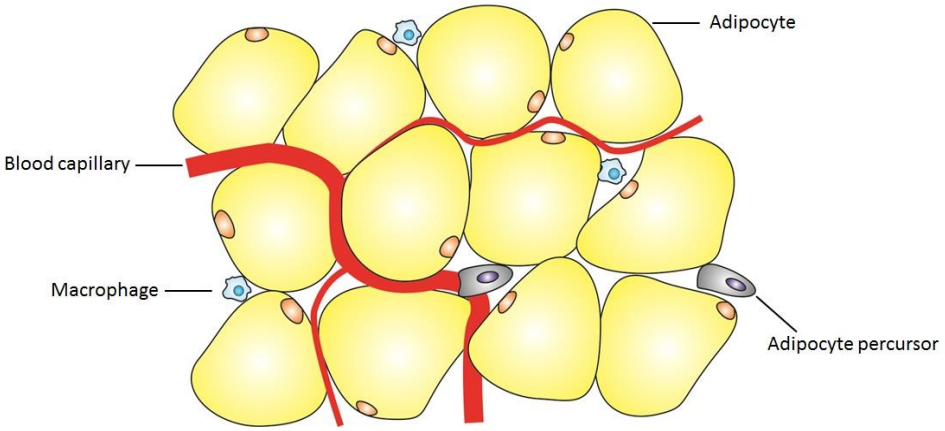

Figure 1.4. Structure of adipose tissue.
White adipose tissue is composed of adipocytes and various cell types such as macrophages and adipocyte precursors and is highly vascularized.

1.4.1.1 Different WAT depots

It is also of importance to acknowledge that WAT is divided into specific regional depots with different biological function and consequently differences in structural organization and cellular size (Fruhbeck, 2008; Gesta *et al.*, 2007; Giorgino *et al.*, 2005). It is thought, that the total adipose tissue mass is less important for the risk of developing obesity-associated diseases than the distribution of fat between these depots (Bjorndal *et al.*, 2011; Giorgino *et al.*, 2005). Although the subcutaneous and visceral adipose tissues are the largest depots in humans, retroperitoneal and epididymal fat are the largest in rodents and for this reason we chose to focus our study on them. Retroperitoneal adipose tissue in rats is located along the dorsal wall of the abdomen, surrounding the kidney. This tissue is known to express high amounts of the lipogenic transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element-binding protein 1 (SREBP1c), lipolysis-related genes and a lower levels of fatty-acid oxidation-related genes compared to mesenteric and subcutaneous adipose tissue (Palou *et al.*, 2009). Epididymal fat is found around the testis in males and around ovaries in females. This depot has also been shown to express more of some lipogenic transcription factors and differences exist between genders (Bjorndal *et al.*, 2011).

1.4.1.2 Storage function, lipolysis and lipogenesis

TG storage is the most important function of adipose tissue as it is vital for survival, particularly in the fasting state. After a meal, energy storage occur in liver and WAT, via esterification of free fatty acid (FFA) and lipogenesis, when levels of insulin, glucose and lipids rise. On the other hand, in the fasted state, glycogen breakdown occurs, lipolysis is stimulated, intracellular lipases activated and adipocytes release FFA and glycerol (Large *et al.*, 2004; Viscarra *et al.*, 2013) (Figure 1.5).

Adipose triglyceride lipase (ATGL), which is the enzyme responsible for the first step of lipolysis acts by hydrolyzing triglycerides (TGs) to diacylglycerols (DGs), providing substrate for hormone sensitive lipase (HSL). Its importance has been demonstrated *in vivo*, as ATGL knockout mice presented increased fat storage in the form of TGs in different tissues, as well as weight gain (Haemmerle *et al.*, 2006). In turn, HSL is responsible for the hydrolysis of stored DG to monoacylglycerol (MAG), being the rate limiting step. HSL activity is actively regulated by cathecolamines through the lipolytic cascade and negatively regulated by insulin. Insulin antagonizes the cathecolamine-induced activation of HSL, a process referred to as the anti-lipolytic effect of insulin (Degerman *et al.*, 1990). This inhibitory effect of insulin is believed to be physiologically the most important mechanism by which the rate of adipocyte lipolysis is reduced/regulated. In this state, perilipin A, the protein responsible for coating the fat droplets, protect the energy stores by blocking fat hydrolysis. When lipolysis occurs, perilipin A is phosphorylated by protein kinase A (PKA). In turn the hyper-phosphorylated perilipin recruits and organizes the activation of the lipolytic machinery (Brasaemle *et al.*, 2009; Skinner *et al.*, 2013). When FFAs and TGS increase in circulation, they become the major energy source for peripheral tissues (Bjorndal *et al.*, 2011). Nonetheless, when this happens in excess, it causes impairment in insulin signaling and consequently glucose intolerance and insulin resistance. This will later translate into type 2 diabetes, non-alcoholic fatty liver and cardiovascular complications, although the molecular mechanisms by which lipids contribute to insulin resistance are not well understood (Czech *et al.*, 2013).

Maintaining the TG storage capacity of adipocytes is of great importance as it keeps lipids away from peripheral tissues, and can promote the release of "good" cytokines, that help maintain insulin sensitivity (Czech *et al.*, 2013). In fact, adipose tissue is an important endocrine organ, responsible for the production of several hormones and adipokines (*e.g.* leptin and adiponectin) but also the pro-inflammatory factors such as Tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (Rosen *et al.*, 2014). These last two have been

associated with the development of insulin resistance, vascular disease and other pathologies in obese patients (Giorgino *et al.*, 2005). After transplantation, pro-inflammatory cytokines are increased and levels of adiponectin decreased in patients suffering from obesity, insulin resistance and *Diabetes mellitus* type 2 (Teplan *et al.*, 2012). On the other hand, adiponectin is one of the most important adipokines, and act as an insulin-sensitizing marker. The main mechanisms by which this occurs seem to be through increased fatty acid (FA) oxidation and inhibition of hepatic glucose production (Kadowaki *et al.*, 2005; Lihn *et al.*, 2005).

After a meal, when glucose is available, the transcription factor carbohydrate-responsive element-binding protein (ChREBP) is activated and translocated from the cytosol to the nucleus (Dentin *et al.*, 2005; Kawaguchi *et al.*, 2001) binding to carbohydrate responsive element (ChRE) present in the promoter regions of glycolytic and lipogenic genes (Ishii *et al.*, 2004). Meanwhile, insulin regulates SREBPs, namely SREBP-1 (isoforms -1a and - 1c) responsible for fatty acid metabolism. SREBP-1c is the predominant isoform in most adult metabolic tissues such as liver and adipose tissue (Jeon *et al.*, 2012). ChREBP and SREBP modulate the expression of genes required to maintain cellular lipid homeostasis (Osborne *et al.*, 2009), namely Acetyl-CoA carboxylases 1 and 2 (*ACC1* and *ACC2*), fatty acid synthase (*FASN*) and Stearoyl-CoA desaturase 1 (*sdc1*) (Denechaud *et al.*, 2008).

ACC enzymes also exist in two isoforms; ACC1 is mainly responsible for the synthesis of TGs and phospholipids while ACC2 is a potent inhibitor of FA oxidation through allosteric binding to carnitine palmitoltransferase (CPT1). ACC1 is expressed at higher levels in liver and adipose tissues than in other tissues while ACC2 is more expressed in heart and skeletal muscle (Wakil *et al.*, 2009). Fatty acid synthase (FAS) is the next step in this pathway. This enzyme is present in most tissues, including adipose tissue and liver (Clarke, 1993). FAS and ACC1 act together, to control the lipogenic flux to convert acetyl CoA and malony CoA into palmitate (Fernandez-Real *et al.*, 2010; Hillgartner *et al.*, 1995; Semenkovich, 1997). In addition, FAS gene expression is also regulated by hormones (*e.g.* insulin) and nutrients. Insulin, in particular, increases its rate of gene transcription in murine cell lines and primary human adipocytes (Claycombe *et al.*, 1998; Wang *et al.*, 2004) as well as its enzymatic activity, in order to produce TG for storage. However, in the state of insulin resistance, FAS is inhibited and the fatty acid pathway is impaired (Fernandez-Real *et al.*, 2010). Lipin 1 is a phosphatidic acid phosphatase 1 (PAP1) enzyme, responsible for the conversion of phosphatidate to diacylglycerol. Lipin 1 mRNA levels in adipose tissue are associated with alterations in metabolism, namely its down regulation causes lipodystrophy, while its up-regulation causes increased TG content per cell and expression of lipogenic

genes. Moreover, lipin levels in adipose tissue influence fat storage capacity of the adipocyte (Phan *et al.*, 2005), without changing insulin sensitivity. Another downstream enzyme is stearoyl-CoA desaturase 1 (SCD1), from the Delta-9 fatty acid desaturase family that exist in the membrane of the endoplasmic reticulum, responsible for the conversion of saturated long-chain fatty acids into monounsaturated fatty acids, which are major components of TGs. Liver and adipose tissue are the predominant sites of SCD gene expression, that respond to diet, hormones and environmental factors (Jones *et al.*, 1998; Kim *et al.*, 2002; Liu *et al.*, 2011; Miller *et al.*, 1997; Ntambi, 1992; Waters *et al.*, 1994).

Moreover the actions of lipoprotein lipase (LPL) are essential to the hydrolysis of the TG core of circulating TG-rich lipoproteins, chylomicrons, and VLDL. This multifunctional enzyme exists in many tissues, including adipose tissue, cardiac and skeletal muscle, and also islets, and macrophages. Furthermore, FA and monoacylglycerols are in part taken up by tissues through CD36 (FA translocase). This tissue-specific expression and regulation of LPL and CD36 have been shown to have major metabolic consequences on macronutrient fuel partitioning, energy homeostasis, insulin action, and lipoprotein metabolism. The enzymatic activity of LPL is regulated in a complex manner in response to energy requirements and hormonal changes. Increasing evidence suggests that LPL is regulated at the transcriptional, posttranscriptional and translational levels in a tissue-specific manner (Wang *et al.*, 2009). Moreover, CD36 deletion impaired FA uptake by key metabolic tissues, and increases plasma FA and TG (Hajri *et al.*, 2002; Su *et al.*, 2009). The final step of TG synthesis is through the conversion of DG to TG in a reaction catalyzed by diacylglycerol acyltransferase (DGAT). DGAT1 is a member of the mammalian ACAT gene family, localized in the endoplasmic reticulum and expressed in adipose tissue, small intestine, mammary gland, liver and muscle (Cases *et al.*, 1998; Coleman *et al.*, 2004; Shi *et al.*, 2009). The importance of TG synthesis is exemplified by severe insulin resistance in patients with lipodystrophy, a genetic condition characterized by defective TG synthesis and storage in adipose tissues (Agarwal *et al.*, 2003). Whereas excess TG accumulation in adipose tissue leads to obesity, ectopic storage of TG in non-adipose tissues such as liver and skeletal muscle is associated with insulin resistance (Shi *et al.*, 2009).

Peroxisome proliferator-activated receptor (PPAR) is a subfamily of nuclear hormone receptors (Desvergne *et al.*, 1999; Lee *et al.*, 2003; Monsalve *et al.*, 2013), whose function as ligand-activated transcription factors is to regulate various biological processes, namely the expression of large numbers of genes involved in regulating the intermediary metabolism of glucose and lipids, adipogenesis, insulin sensitivity, immune response, cell

growth, and differentiation (Desvergne *et al.*, 1999; Fajas *et al.*, 2001; Feige *et al.*, 2006; Guan *et al.*, 2001; Monsalve *et al.*, 2013; Willson *et al.*, 2001). PPAR exists in three isoforms PPAR-α, PPAR-β/δ, and PPAR-γ (Dreyer et al., 1992), being the last one responsible for promoting FFA storage in mature adipocytes and activate glucose transporter 4 (GLUT4) transcription, facilitating increased FFA synthesis from glucose (Bjorndal *et al.*, 2011; Boon Yin *et al.*, 2008; Wu *et al.*, 1998).

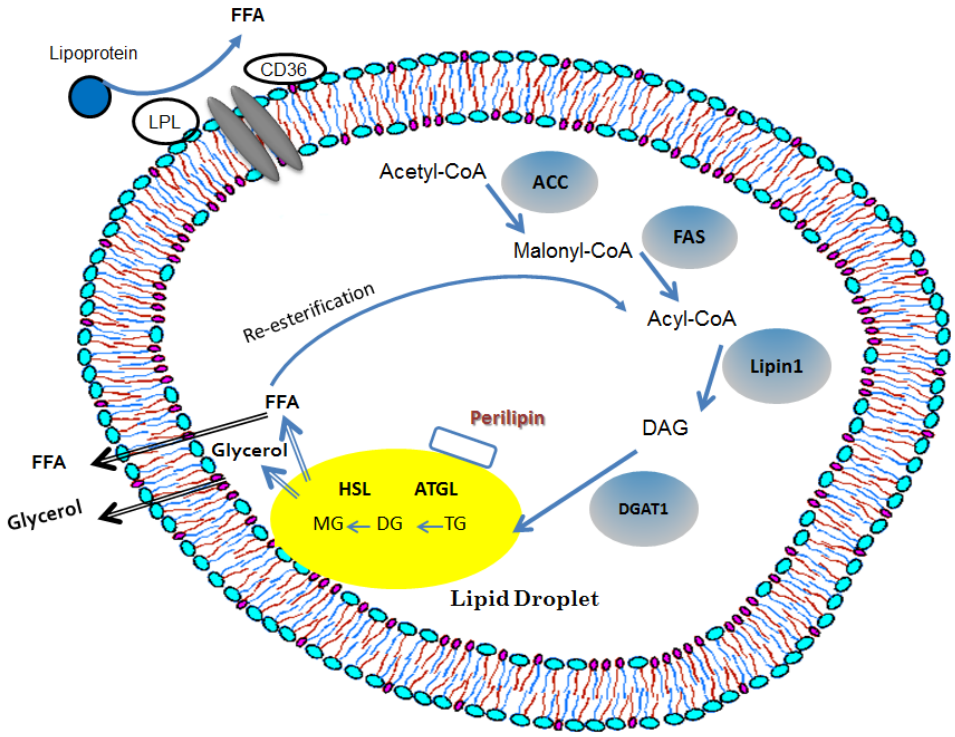

Figure 1.5. Lipolysis and lipid storage in adipocytes.
Free fatty acid (FFA); lipoprotein lipase (LPL); fatty acid translocase (CD36); acetyl-CoA carboxylase (ACC); fatty acid synthase (FAS); diacyl-glycerol (DG); diglyceride acyltransferase 1 (DGAT1); triglycerides (TG); monoacylglycerol (MG); adipose triacylglycerol lipase (ATGL); hormone-sensitive lipase (HSL)

1.4.1.3 Insulin signaling in adipose tissue

Adipose tissue also has the capacity to respond to insulin and increase glucose uptake. This process begins with the binding of insulin to its cell surface receptor (IR), activating the intrinsic tyrosine kinase and its auto-phosphorylation. This action generates docking sites for insulin receptor substrate proteins (IRS-1 – IRS-4). Several downstream signaling pathways are activated, which include p85 regulatory subunit of phosphoinositide 3-kinase (PI3-kinase), which ultimately activates Akt (also called PKB). This last step

triggers numerous pathways that control carbohydrate and lipid homeostasis. But most importantly, it activates phosphoinositide-dependent kinase-1(PDK1) and Protein kinase C (PKC), phosphorylating intracellular vesicles containing the glucose transporters – GLUT4 – that translocate from intracellular membrane vesicles and fuses with the plasma membrane. Another AKT substrate is AS160, a 160 kDa protein, which include GTPase-activating domain for small G-proteins (Rabs), that when phosphorylated have an important role in the activation of GLUT4 vesicle exocytosis (Bruss *et al.*, 2005; Sano *et al.*, 2003; Zeigerer *et al.*, 2004). Five minutes of exposure to a physiological concentration of insulin is enough to stimulate the translocation of GLUT4-containing vesicles. Therefore, glucose uptake is the rate-limiting step in glucose utilization and/ or storage and as such has a key role in the maintenance of glucose homeostasis (Leto *et al.*, 2012; Summers *et al.*, 2000). However, dysregulation of the autonomic nervous activity, catecholamines, adipokines and immunosuppressive agents might affect phosphorylation of proteins involved in insulin signaling and therefore impair glucose uptake in insulin sensitive tissues (Buren *et al.*, 2005; Lundgren *et al.*, 2004)

1.4.2 Muscle

Skeletal muscle or striated muscle is the muscle attached to the skeleton. It is composed of thousands of cylindrical muscle fibers, bound together by connective tissue, vascularized and innervate, and its contraction is voluntary (Figure 1.6).

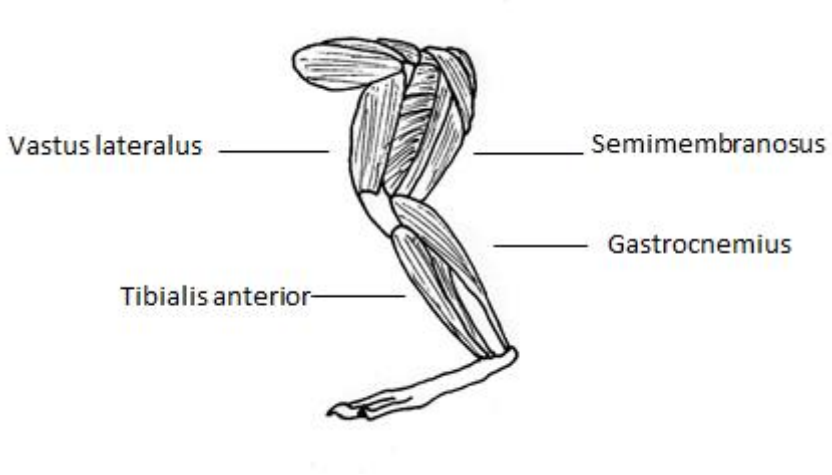

Figure 1.6. – The anatomy of the rat leg skeletal muscle.

Skeletal muscle has two energy depots – glycogen and TGs, both stored in the cytoplasm (Langfort *et al.*, 2003). Glycogen can rapidly be converted into glucose, when energy is required, through glycolysis, an anaerobic process, which produces three ATP and two lactic acid molecules. TGs, stored in lipid droplets, can be mobilized by catecholamines and oxidized in the mitochondria. Due to its mass, skeletal muscle is the major contributor to whole-body energy metabolism, it accounts for 75% of whole-body insulin stimulated glucose uptake and storage (Corcoran *et al.*, 2007; DeFronzo *et al.*, 1981; Roden, 2004; Shulman *et al.*, 1990).

1.4.2.1 Insulin signaling in muscle

As mentioned above, skeletal muscle is the main tissue responsible for insulin-stimulated glucose uptake. In a process very similar to that describe for adipose tissue, insulin binds to its receptor and activates a cascade that will phosphorylate Akt, the protein kinase responsible for the activation of glycogen synthesis, protein synthesis, and GLUT4 translocation to the cell surface (Figure 1.7), thereby increasing glucose transport (Bruss *et al.*, 2005; Czech *et al.*, 1999; Lawlor *et al.*, 2001; Pessin *et al.*, 2000; Whiteman *et al.*, 2002).

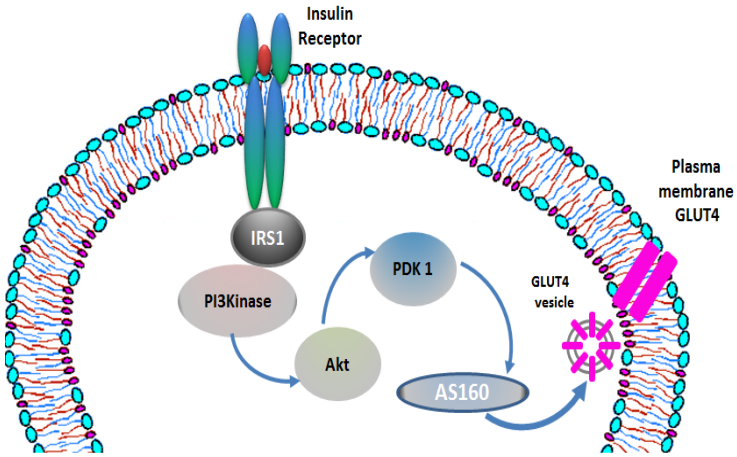

Figure 1.7. – Insulin signaling pathway.
Insulin receptor substrate 1 (IRS1); phosphoinositide 3-kinase (PI3K); Protein kinase B (Akt/PKB); phosphoinositide-dependent kinase 1(PDK1); Akt substrate of 160 kDa (AS160); glucose transporter 4 (GLUT4).

Insulin stimulation is responsible for multiple tyrosine phosphorylation events, which are controlled by protein-tyrosine phosphatase (PTP). One specific PTP is expressed

in all insulin-responsive tissues on the cytoplasmic face of the endoplasmic reticulum – PTP1B (Frangioni *et al.*, 1992). It has been reported than in cases of increased PTP activity, namely PTP1B, insulin resistance develops through inhibition of insulin-stimulated phosphorylation of the IR and IRS-1 (Byon *et al.*, 1998; Goldstein *et al.*, 1998; Klaman *et al.*, 2000).

The development of metabolic complications, like insulin resistance in obesity or diabetes, affects the whole-body glucose homeostasis. FFA can directly inhibit glucose transport and phosphorylation in skeletal muscle. In fact, Dresner *et al.* (1999) described the inhibition of IRS-1 associated PI3K, when FFA increased in circulation. Moreover, the tyrosine phosphorylation of IRS-1 was also impaired, while Akt/PKB phosphorylation was maintained intact (Kruszynska *et al.*, 2002; Roden, 2004). On the other hand, insulin resistance in muscle can also promote dislipidemia by stimulating the conversion of energy derived from ingested carbohydrate into hepatic *de novo* lipogenesis and increased VLDL production. In fact, when the IR gene is inactive in mice, TGs increase in circulation and adiposity increases as a consequence of specific insulin resistance.

1.4.3 Liver

The liver, the largest organ in the body, is structurally and functionally very complex. It is responsible for numerous actions like carbohydrate and lipid metabolism, as well as protein synthesis; while some of these products are meant for storage, others are released into the circulation, to help maintaining homeostasis (Malarkey *et al.*, 2005).

Mice and rats each have 4 liver lobes: median (or middle), left, right, and caudate (Figure 1.8). The rat and human liver have two blood supply sources, *i.e.* a portal and hepatic venous systems (Kogure *et al.*, 1999; Malarkey *et al.*, 2005). The portal vein supplies about 70% of the blood flow and 40% of the oxygen while the hepatic artery supplies 30% of the flow and 60% of the oxygen (Malarkey *et al.*, 2005). The portal blood come from the mesenteric, gastric, splenic, and pancreatic veins and is delivered to the liver, through its right and left sides. The gastrointestinal tract and spleen offer an incomplete mixing of blood with a variation in delivery of various nutrients, toxins, and other elements to the liver lobes (called portal streamlining) (Haywood, 1981; Malarkey *et al.*, 2005).

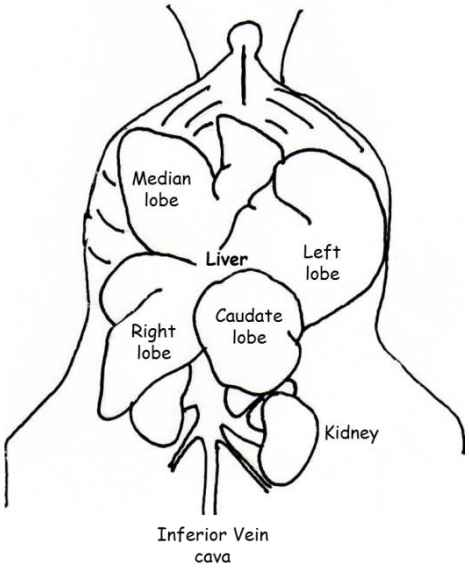

Figure 1.8. – The anatomy of the rat liver.

Hepatic glucose metabolism plays an important role in glucose homeostasis by maintaining a balance between the uptake and storage of glucose and the release of glucose in response to the nutritional status.

After a meal, the blood transported through the hepatic portal vein directly from the gastrointestinal tract is rich in glucose. This stimulates insulin to bind to its receptor and cause the phosphorylation of intracellular substrate such as IRS-1, IRS-2 and Akt. Glucose is taken up by the primary hepatic liver transporter, a low affinity, high capacity transporter expressed at high levels on the sinusoidal membranes of hepatocytes and non-insulin sensitive glucose transporter (GLUT2) (Eisenberg *et al.*, 2005; Leturque *et al.*, 2005). Moreover it also activates glucokinase (GK), which occur through the rapid mobilization of GK from the nucleus to the cytoplasm and glucose is phosphorylated to Glucose-6 phosphate (G6P).

When the concentration of G6P increases it acts synergistically with glucose, to promote the inactivation of glycogen phosphorylase and the activation of glycogen synthase leading to an increase in glycogen storage in the liver (Agius, 2008). It has also been reported that hyperinsulinemia can increase glycogen synthesis by activating glycogen synthase and inhibiting glucagon, the glycogenolytic protein (Cohen *et al.*, 1978).

1.4.3.1 Gluconeogenesis

Gluconeogenesis is the production of glucose from non-carbohydrate substrates (*e.g.* pyruvate, lactate, gluconeogenic amino acids and glycerol) and includes anaplerotic fluxes from the tricarboxylic acids (TCA) cycle. It is promoted in the post-absorptive state, as insulin and glucose levels decrease and liver produces glucose, converting it to G6P with the help of Glucose-6-phosphatase (G6Pase). Gluconeogenesis is directly mediated by insulin, that inhibit the expression of key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase, and the upstream transcription factors peroxisome proliferator-activated receptor- coactivator (PGC1-α) and Forkhead box O1 (FOXO1), along with fructose-1,6-bisphosphatase (F1,6Pase) (Barthel *et al.*, 2003). Furthermore, during fasting, glucagon and catecholamines activate PGC1-α, through the cAMP pathway and the cAMP response element-binding protein (CREB) transcription factor, which will in turn activate FOXO1; or as suggested by Ropelle *et al.* (2009), PGC1-α and FOXO1 interact with each other and initiate an important signal transduction pathway that promote the synthesis of glucose in the liver. In the diabetic state or insulin deficiencies, PGC1-α activity is increased and might be the cause of an increase in hepatic glucose production (Liang *et al.*, 2006; Yoon *et al.*, 2001). On the contrary, when insulin causes the phosphorylation of Akt, FOXO1 is excluded from the nucleus, therefore inhibiting its transcriptional activity (Wan *et al.*, 2011), as well as hepatic gluconeogenesis gene expression and glucose output (Haeusler *et al.*, 2010; Li *et al.*, 2007; Liu *et al.*, 2008; Matsumoto *et al.*, 2007; Puigserver *et al.*, 2003; Qu *et al.*, 2006; Wan *et al.*, 2011). Insulin also directly decreases gluconeogenesis by inhibiting adipose tissue lipolysis that reduces both FFA and glycerol release and consequently the availability for the gluconeogenesis process (Figure 1.9). Insulin also inhibits muscle proteolysis, resulting in a decreased availability of gluconeogenic amino acids (Umpleby *et al.*, 1996).

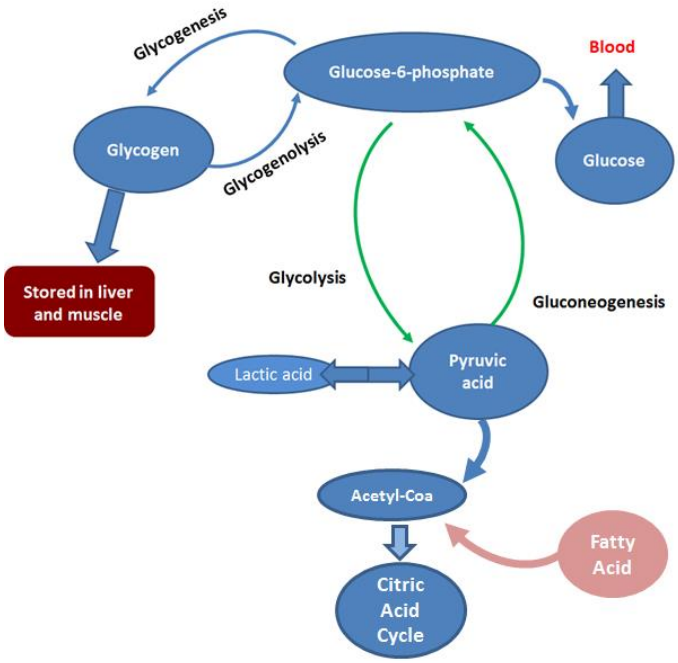

Figure 1.9. Overview of the gluconeogenesis, glycolysis, glycogenesis and glycogenolysis pathway.

1.4.3.2 Hepatic de novo lipogenesis

Substrate supply and hormonal regulation modulates enzyme activity in the liver. In the fed state, insulin rise promote hepatic *de novo* lipogenesis by stimulating FAS and activating pyruvate dehydrogenase and ACC to form acetyl-CoA and malonyl-CoA, respectively. Moreover, insulin also inactivates pyruvate carboxylase and lactate dehydrogenase (Sul *et al.*, 2000; Wang *et al.*, 1998). In addition, after a meal, uptake of excess fatty acids occurs from circulation in proportion to their concentration in blood, and are re-esterified to TGs within the liver, or exported as constituents of VLDL. In post-absorptive conditions, FAS decreases, leading to a reduction of hepatic *de novo* lipogenesis. In parallel, in mitochondria, □-oxidation of fatty acids increases in order to produce energy for the liver (Nguyen *et al.*, 2008).

1.4.3.3 Hepatosteatosis

The liver has a central role in lipid metabolism, being involved in TG synthesis and lipids in circulation. Under normal conditions, the liver receives non-esterified fatty acid (NEFA) and dietary fatty acids flow and produces new fatty acids through lipogenesis. After the esterification step, TGs can be stored as lipid droplets in the liver, secreted into circulation as VLDL and/or be conveyed to the β-oxidation pathway (Postic *et al.*, 2008). Nonetheless, if the proper function is altered, accumulation of lipid droplets into the

hepatocytes results in hepatic steatosis (Nguyen *et al.*, 2008; Postic *et al.*, 2008). The relationship between fatty acids in the liver and insulin resistance is known for a long time, nonetheless it remains to be clarified whether it is the insulin resistance that causes the accumulation of lipids in excess in the liver or if it is the accumulation of TGs (or their intermediates) that causes the hepatic or systemic insulin resistance (Postic *et al.*, 2008). Liver steatosis may also be caused by deregulation of lipid metabolism, by some of its intervenient factors such PPAR-γ, SREBP1, FAS or ACC in certain conditions.

1.5 Nuclear magnetic resonance (NMR) and stable isotopes to monitor effects of immunosuppressive agent on glucose and lipid metabolism

Understanding how changes in glucose and lipid metabolism can affect the success of the transplant and the quality of life of patients is an important objective. Metabonomics studies allow the comparison of metabolite profiles among different tissues or body fluids by measuring large arrays of metabolites; this approach can be used to assess effects of different treatments on metabolic pathways or biomarkers for metabolic diseases (Kim *et al.*, 2010). Human metabolom database has 41.514 entries with 4.229 identified in serum alone (Psychogios *et al.*, 2011). This technology is promising because changes in metabolite profile may be easier to detect at an early phase, well before histologic and pathophysiologic changes occur (Christians *et al.*, 2008). Metabolites can be detected by mass spectroscopy and by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (Kim *et al.*, 2010). In rat models and human patients, changes in endogenous metabolites have already been observed by these techniques in blood and urine after CsA administration (Klawitter *et al.*, 2010).

In addition to monitoring endogenous metabolites by observing the ^{1}H and ^{31}P nuclei, NMR can also be used to study the metabolism and incorporation of stable isotopes such as ^{13}C and ^{2}H tracers into metabolic intermediates and end-products. Measuring the incorporation of ^{2}H from deuterated water ($^{2}H_2O$) and quantifying the ^{2}H-enrichment of glucose, glycogen and TG by ^{2}H NMR reflects the activities of gluconeogenesis, glycogenesis and de novo lipogenesis, respectively (Delgado *et al.*, 2013; Jones *et al.*, 2001; Soares *et al.*, 2009).

1.6 Known metabolic effects of calcineurin and mTOR inhibitors on peripheral tissues

As mentioned before, CsA and SRL therapies are associated with NODAT and dyslipidemia; what follows is a summary of some of their known effects on the peripheral tissues studied, *i.e* adipose tissue, muscle and liver.

1.6.1- Effects of calcineurin and m-TOR inhibitors on glucose metabolism

Calcineurin inhibitors have been associated with deleterious impact on pancreatic β-cells by decreasing the normal secretion of insulin and inhibiting insulin gene expression (Oetjen *et al.*, 2003; Polastri *et al.*, 2002). Moreover reduced β-cell mass, insulin granule amount and higher apoptosis in β-cell lines, have been observed in rodent and human islets (Ajabnoor *et al.*, 2007; Bugliani *et al.*, 2009; Drachenberg CB, 1999; Hahn *et al.*, 1986; Hernandez-Fisac *et al.*, 2007; Lucke *et al.*, 1991). They have also been shown to impair both basal and glucose-stimulated insulin secretion and inhibit calcineurin activity in β-cells (Øzbay *et al.*, 2012). Although early data did not link SRL to toxicity in pancreatic β-cells, new evidence from *in vitro* and in *vivo* experiments, has shown differently (Barlow *et al.*, 2013). SRL reduce β-cell mass *in vivo* (Houde *et al.*, 2010) and affect cell survival, increasing levels of apoptosis, as well as their function and viability in murine and human β-cells (Barlow *et al.*, 2013; Barlow *et al.*, 2012; Bell *et al.*, 2003; Fraenkel *et al.*, 2008; Yang *et al.*, 2012).

Blood glucose concentration and peripheral insulin sensitivity are also affected by immunosuppressive agents (Subramanian *et al.*, 2007).

In rats, CsA promotes glucose intolerance, attributed to low levels of plasma insulin and/or impaired insulin action and increased glycogenolysis (Delgado *et al.*, 2012). Immunosuppressive agents (IA) studies in humans are usually limited by study design with confounding factors, such as the use of other agents. Nonetheless, it has been demonstrated that pharmacological doses of CsA impairs the 2nd phase of insulin secretion, without changing the 1st phase of secretion (Hjelmesaeth *et al.*, 2007; Robertson *et al.*, 1989). While calcineurin inhibitors have been involved in the inhibition of the phosphorylation of the IR, it has not been associated with any change in expression or phosphorylation of proximal insulin signaling cascade proteins (Pereira *et al.*, unpublished data; Shivaswamy *et al.*, 2013).

SRL also causes insulin resistance and severe glucose intolerance attributed not only to reduced β-cell mass but mostly to increased hepatic gluconeogenesis (Fraenkel *et al.*, 2008; Houde *et al.*, 2010; Lamming *et al.*, 2012). In this case, expression of gluconeogenic

genes - PEPCK and G6Pase was increased in liver, associated with an impaired pyruvate tolerance test, which clearly points to an inability to suppress gluconeogenesis (Houde *et al.*, 2010; Lamming *et al.*, 2012). Furthermore, other have shown that SRL reduces glucose uptake and Akt/PKB phosphorylation in humane mature adipocytes and rat insulin sensitive cells (Kumar *et al.*, 2010; Pereira *et al.*, 2012; Sarbassov *et al.*, 2006; Shivaswamy *et al.*, 2013). Blättler *et al.* (2012) also observed that SRL reduces insulin resistance, caused by the suppression of insulin/IGF signaling, and by other genes associated with this pathway, such as IGF1-2, IRS-1-2, and Akt1-3. Alterations in the insulin signaling pathway can affect normoglycemia (Rhodes *et al.*, 2002). In contrast, some studies suggest that SRL improves insulin-stimulated glucose uptake and Akt/PKB phosphorylation in L-6 muscle cells, 3T3-L1 cells and in differentiated adipocytes (Berg *et al.*, 2002; Tremblay *et al.*, 2005; Tremblay *et al.*, 2001).

1.6.2 - Effects of calcineurin and mTOR inhibitors on lipid metabolism

The association of calcineurin and m-TOR inhibitors with dyslipidemia is well known. In rodents, the administration of CsA increases the plasma levels of cholesterol, TG, VLDL and LDL cholesterol, as well as ratios of total cholesterol/ HDL, apolipoprotein B (apoB) and LDL /HDL (Espino *et al.*, 1995; López-Miranda J, 1992). The increase in TG and cholesterol in circulation was associated with a decrease in plasma LPL activity (Espino *et al.*, 1995; López-Miranda J, 1992) and LPL abundance in skeletal muscle and adipose tissue (Vaziri *et al.*, 2000). Moreover, the inhibition of hepatic 26-hydroxylase and the down regulation of hepatic cholesterol 7 α-hydrolase, the rate limiting enzymes that allow the reduction of the level of cholesterol in hepatocytes by converting them to bile acids, result in increased serum levels of cholesterol (Hulzebos *et al.*, 2004; Vaziri *et al.*, 2000; Watt, 2011). Furthermore, the ability of CsA to bind to LDL cholesterol receptors may contribute to the dyslipidemia observed in rodents and humans (Munoz, 1995; Watt, 2011). In addition, CsA effects on hepatic mRNA levels for a number of key genes involved in cholesterol and fatty acid biosynthesis pathways, such as HMG-CoA reductase, LDL receptor, SCD1, squalene synthase, transcriptional factors SREBP 1 and 2, FAS and ACC1 and ACC2 mRNA, may also contribute to increased hepatic TG secretion (Delgado *et al.*, 2012; Wu *et al.*, 1999).

In addition, SRL has also been associated with dyslipidemia after renal transplant, which might result in increased hepatic production of TG rich lipoproteins and/or decreased clearance of them, as well as a reduction in the fractional catabolic rate of apoB-100-

containing lipoproteins (Hoogeveen *et al.*, 2001; Morrisett *et al.*, 2002). SRL also caused a significant increase in cholesterol and TGs, which might be due to increased activity of HSL and inhibition of LPL (Morrisett *et al.*, 2002). As a consequence, lipids are not stored efficiently in adipocytes and FFAs are released into the circulation resulting in increased VLDL. In addition, SRL decreases FFA oxidation, increasing their availability in blood (Morrisett *et al.*, 2002). Lipid metabolism might also be altered at the gene level, with overstimulation of SREBP-1c mRNA and down regulation of PEPCK (Li *et al.*, 2010). Tory R (2008) suggested that increase in cholesteryl ester transfer protein (CETP) activity and suppression of LPL activity after CsA and SRL treatment could be associated with elevated LDL cholesterol levels and hypertriglyceridemia observed in patients (Kontush A, 2006; Zeljkovic A, 2011).

The main goal of post-transplant immunosuppressive therapy is the induction of tolerance to allografts (Smith *et al.*, 2003), which have been successfully accomplished by all the agents referred. However, and despite the fact that CsA and SRL have been used for years in clinic settings, many of their biomolecular mechanisms of action at the tissue and cellular levels are still poorly understood. As long as the precise molecular mechanisms underlying the effects of IAs on glucose and lipid metabolism remain to be elucidated, the most proper immunosuppressive protocols for each specific condition will remain deficient and long-term outcomes may not improve significantly. The clinical follow-up data is yet insufficient, and animal studies focusing on clinically relevant doses of the agents are advisory. Since organ transplantation is increasing and it is happening at younger ages, it is greatly important that the scientific community devotes more resources in trying to understand these molecular mechanisms so that better agents, with fewer side effects, can be developed.

1.7 Scope, aims and outline of the thesis

The overall aim of this thesis was to study the *in vivo* effect of two of the most used IAs, namely cyclosporine A and sirolimus, on glucose and lipid metabolism, in a rat model, in order to understand the molecular mechanism, involved in the development of insulin resistance and dyslipidemia.

The specific aims were to:

- Investigate the effects of CsA and SRL on insulin-stimulated glucose uptake in isolated epididymal adipocytes after either *ex-vivo* or *in vivo* treatment in Wistar rats (*Chapter 3*).

- Investigate the effects of CsA and SRL on the insulin signaling pathway and on genes and proteins involved in glucose metabolism in liver, muscle and adipose tissue after 3 weeks of treatment, in Wistar rats *(Chapter 4)*.

- Investigate the effects of CsA and SRL on lipolysis and expression of genes and proteins involved in lipid metabolism in liver and adipose tissue in Wistar rat after 3 and 9 weeks treatment, in Wistar rats *(Chapter 5)*.

- Investigate the influence of CsA on hepatic *de novo* lipogenesis and its gluconeogenic contributions to hepatic glycogen by measuring triglyceride and glycogen deuterium enrichment from $^{2}H_2O$ enriched body water using ^{2}H-NMR analysis *(Chapter 6)*.

Diabetes mellitus is a widespread and growing public health problem due mainly to the aging of the population and changes in the life style. It is associated with the metabolic syndrome, and consequently with insulin resistance and dyslipidemia; both conditions have also been linked with the use of immunosuppressive therapy. CsA and SRL are two of the immunosuppressive agents used after organ transplantation. The success rate of transplants depend on how both these agents affect glucose and lipid metabolism on peripheral tissues, such as, adipose tissue, muscle and liver. Although used regularly, the molecular mechanisms underlying the effects of these agents remain to be fully elucidated.

Chapter 3 and 4 focus on glucose metabolism. First we evaluate the influence of immunosuppressive agents - CsA and SRL - on glucose metabolism, through the measurement of insulin-stimulated glucose uptake in isolated primary adipocytes. We then studied gene and protein expression levels of factors involved on glucose metabolism as well as the insulin signaling in liver, muscle and adipose tissue.

In chapter 5, we focus on lipid metabolism in adipose tissue and liver, and how it is altered after either short or long-term treatment with CsA and SRL.

Chapter 6 elucidates a novel approach using ^{2}H-NMR analysis to determine the influence of CsA in lipid and glucose metabolism

The chapter 7 includes the major conclusions that may be addressed from the developed work as well as future perspectives.

CHAPTER 2

Material and Methods

2.1 Animals and treatments

Male Wistar rats (Charles River Lab. Inc., Barcelona, Spain) 10 weeks old, weighing ≈300 g, 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 chow (IPM-R20, Letica, Barcelona, Spain) and free access to tap water. All animal care and experimental procedures were conducted according to the National and European Communities Council Directives on Animal Care (86/609/EEC).

For experiments in chapter 3, 4 and 5, animals were randomly divided into 3 groups: Vehicle (10% orange juice), CsA – 5 mg/Kg body weight (BW)/day of Sandimmune Neoral® and SRL – 1 mg/kg BW/day of Rapamune® dissolved in orange juice. Treatments were performed daily by oesophageal gavage for 3 or 9 weeks, and body weight was monitored weekly throughout the study.

For experiments in chapter 6, animals were randomly divided into two groups (n=8/group): Vehicle (orange juice) and CsA – 15 mg/Kg body weight (BW)/day of Sandimmune Neoral® (Novartis Pharma, Lisbon, Portugal) and treatments were performed daily by oesophageal gavage for 2 weeks.

At the end of treatments, rats were anesthetised i.p. with 2mg/Kg BW of a 2:1 (v:v) Ketamine (50mg/ml) (Ketalar®, Parke-Davis, Pfizer Labs Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largatil®, Rhône-Poulenc Rorer, Vitória labs, Amadora, Portugal).

For experiments in chapter 4, in each group, 8 animals received a bolus of insulin - Actrapid (i.p 10 U/kg) and were sacrificed 10 minutes later, in order to study insulin action *in vivo*, in the insulin sensitive tissues. The other 8 animals received saline as a control.

Blood samples were collected by venepuncture from the jugular vein using needles with no anticoagulant, for serum sample collection or with appropriate anticoagulant (ethylenediamine tetraacid – EDTA) for plasma samples for further analysis. Liver, muscle, perirenal and epididymal fat were harvested and frozen in liquid nitrogen for further analyses.

2.2 Biochemical parameters

Glucose, TG and total-cholesterol levels were measured in serum through automatic validated methods and equipment (Hitachi 717 analyser, Roche Diagnostics Inc., Holliston, MA, USA). NEFAs were measured using a FFA kit (NEFA C-test Wako, Wako Pure Chemicals, Neuss, Germany). Serum insulin and C-peptide were determined using an enzyme-linked immunoadsorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden). Insulin resistance was estimated by the homeostasis model assessment–insulin resistance (HOMA-IR), using the formula: (fasting insulin [µU/ml] X fasting glucose [mmol/L])/22,5 (Mari *et al.*, 2001). CsA and SRL trough blood concentrations were assessed by automatic methods (Flex reagent, Dimension®RxL, Siemens, Germany).

TGs in liver and muscle were measured using a Triglyceride Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) after Folch extraction. Lipid mass was normalized to tissue weight.

2.2.1 – Glucose clearance rate in the urine

Animals (n=6/group) were housed in metabolic cages during 24 hours and received tap water and food *ad libitum.* The 24 hour urine was collected, volume was measured and glucose was assessed in Cobas Integra® 400 plus (Roche Diagnostics Inc, MA, USA), in order to calculate the glucose clearance rate.

2.3 Glucose and insulin tolerance tests

Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed at the end of treatments. A glucose solution was injected (i.p. 2 g/kg BW) after a 16h fast for the GTT and for the ITT, a solution of insulin (i.p 1 U/kg BW; Actrapid) diluted in saline 0.9% (w/v) after 6h of food removal. Blood was collected from the tail vein prior to (0 min) and

at the various times after injection. Glucose blood levels were measured using a glucometer (AccuChek Active, Roche Diagnostics®, Indiana, USA).

2.4 Adipocyte isolation and measurement of cell size, weight and number

Adipocytes were isolated from adipose tissue according to methods previously reported (Carvalho *et al.*, 2000; Hirsch *et al.*, 1968; Smith, 1971). After sacrifice epididymal adipose tissue was harvested, cut in small pieces and was digested with collagenase type II (from *Clostridium histolyticum*) in Krebs Ringer HEPES (KRH) buffer supplemented with 6 mM glucose, 0.15 µM adenosine, pH 7.4 in a gently shaking water-bath at 37ºC for 30 min. KRH buffer was prepared with 4% Bovine Serum Albumin (BSA), 140 mM Sodium Chloride (NaCl), 4.7 mM Potassium Chloride (KCl), 1.25 mM Magnesium Sulfate ($MgSO_4$), 1.26 mM Calcium Chloride (CaCl), 5.8 mM Sodium Phosphate (NaH_2PO), 200 nM adenosine deaminase and 25mM Hepes (Sigma Chemical Co). Isolated adipocytes were filtered through a 250 µm nylon mesh, washed four times and suspended in KRH buffer (without glucose).

After obtaining the final cell solution (1:10), 500 µl 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 and 1ml of pure H_2O. Tubes were shaken vigorously and left for 2h at room temperature before centrifuging for 5 min at 3000g. The upper phase was placed in previously weighed vials and allowed to dry in the hood. Afterwards the final weight obtained represents the TG mass in 500 µl cell suspension. Experiments were performed in triplicates.

Moreover, the average diameter of 100 adipocytes isolated from the animals was measured using a B1 series microscope (System Microscopes-Motic) with a 40X ocular with an internal ruler. About 150-200 µl of cell solution (1:10) was placed on previously prepared slides (fixed with Silicon Oil and heated to 100 ° C for 1 hour).

The weight of adipocytes was calculated based on the assumption that fat cells are spherical and their density is that of triolein (0.95 g/cm^3), using the formula: $V= \pi/6(3\sigma^2+ X^2)X$ (V= volume; σ= variance of mean cell size ; X=mean cell size).

Finally, the cell number per 500µl of solution was calculate using the formula: [(2.45 x TG mass)/cell weight] x 10^6.

2.4.1 Insulin-stimulated glucose uptake

Insulin-stimulated ^{14}C-glucose uptake in the isolated rat adipocytes was assessed as previously reported (Carvalho *et al.*, 2000). Adipocytes were isolated as mentioned above, and for the *ex vivo* experiments, adipocytes were pre-incubated for 30 min in the presence or absence of CsA (0.5-30 μM) and SRL (1-250 μM). After this time, adipocytes were incubated for further 10 min with or without 10 nM of insulin. Finally, D-[U-^{14}C] glucose (0.30 mCi/L, final concentration 0.86 μM) was added for another 30 min. Cell suspension was then transferred to pre-chilled tubes, containing silicone oil, in order to promote the separation of the cells from the buffer by centrifugation for 5 min at 3000g. Cell-associated radioactivity was analyzed by liquid scintillation counting in a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer Life, Shelton, CT, USA), to determinate the rate of trans-membrane glucose transport (Kashiwagi *et al.*, 1983). Experiments were performed in triplicates. 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) (c.p.m.- counts per minute) (Yu *et al.*, 1997).

2.4.2 Lipolysis

Lipolysis was performed as previously described (Pereira *et al.*, 2013). Adipocytes were isolated in a similar way as mentioned above but always using KRH buffer supplemented with 6 mM glucose, 0.15 μM adenosine, pH 7.4. The diluted (1:10) suspension of isolated adipocytes from CsA and SRL-treated rats was supplemented with isoproterenol (1 μM) and insulin (1000 μU/ml) and incubate in a gentle shaking water bath at 37ºC for 1 h. Cell suspension was then transferred to pre-chilled tubes, containing silicone oil, in order to promote the separation of the cells from the buffer by centrifugation for 5 min at 2000 rpm 15ºC. The medium was separated from the adipocytes, and glycerol levels were measured in the extracellular medium using a colorimetric absorbance Adipocyte Lipolysis Assay Kit (Glycerol Detection – Zen Bio, NC, USA) and used for estimations of the effects of CsA and SRL on lipolysis.

2.5 Staining

Perirenal adipose tissue samples were also fixed in 10% phosphate buffered formalin and embedded in paraffin. Thin sections (3 µm) were mounted on glass slides and dyed with hematoxylin/eosin. Digital images of tissue slices were captured using a microscope Axioskop 2 plus with Digital Camera Axiocam HRC (Zeiss, Jena, Germany).

Oil Red O staining was performed on frozen liver and muscle sections (10 µm) previously fixed in 10% formalin for 5 min as previously described (Kumar *et al.*, 2010). Briefly, slides were rinsed 3 times with propylene glycol (85%) and then placed in 0.5% Oil Red O stain solution in propylene glycol for 30 min before being rinsed with 85% propylene glycol for 1 min and counterstained with hematoxylin. Thereafter, the slides were washed with distilled water and mounted with aqua mounting medium (Sigma, St. Louis, MO, USA). Sections were observed with an Axioskop 2 plus microscope fitted with an Axiocam HRC digital camera.

2.6 RNA extraction and cDNA synthesis

RNA from perirenal adipose tissue, liver and muscle was extracted according to the manufacturer's instructions using RNeasy Mini Kit (QIAGEN Sciences, Germantown, MD, USA). The extracted RNA was quantified at 260 nm in a Nanodrop spectrophotometer (Thermo Scientific, USA) and cDNA was synthesized using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems; Forest City, CA, USA) as follow: 2µl of 10X RT Buffer, 0.8 µl of 25X dNTP Mix, 2 µl of 10X RT random primers, 1 µl of MultiscribeTM Reverse Transcriptase and 4.2 µl of nuclease free H_2O to 10 µl of RNA (1 µg) sample.

2.6.1 Real-time PCR

To analyse genes of interest in different tissues, real-time polymerase chain reaction (PCR) was performed. For each reaction 10 µl volume was used containing 2.5 µl cDNA, 5 µl 2X Syber Green Supermix, 1 µl of each primer (250 nM) and 0.5 µl of H_2O PCR grade were added. Primer sequences are given in Table 2.1. Relative mRNA levels changes were measure with a CFX Manager ^{TM} version 2.0 Real-Time PCR detection system (Bio-Rad laboratories, Hercules, CA, USA). The measured fluorescent reflects the amount of amplified product in each cycle. The cycle number at which enough amplified product accumulates to yield a detectable fluorescent signal, is called the threshold cycle (Ct). Ct

value is measured in the exponential phase when reagents are not limiting. Final calculations were based on the delta CT values. Relative mRNA levels for the different genes were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and TATA –binding protein (*TBP*) mRNA levels for each sample, that were selected based on our previous results demonstrating that it does not changed under these conditions.

Table 2.1 – Forward and Reverse primers sequences used for RT-PCR

Primer	**5' – 3' sequence (foward; reverse)**
Acetyl CoA Carboxylase *(ACC1)*	F:AAGGCTATGTGAAGGATGTGG R: GAGGTTAGGGAAGTCATCTGC
Fatty acid synthase *(FASN)*	F:CTAAGACTGAAGCATCTG R: ATACAGAGAACGGATGAG
Lipoprotein lipase *(LPL)*	F: CAGCAACATTATCCAGTG R: GTAGTTAAATTCTTCCTCCAA
Peroxisome proliferator-activated receptor gamma *(PPARG)*	F: CCACACTATGAAGACATC R: CTACTTTGATCGCACTTT
Hormone sensitive lipase *(HSL)*	F: GGGCAGAAGGATGAAACC R: GACACAGAGGTAGAACTTGG
Perilipin A *(PLIN)*	F: GACCATCACAGTCAGGTT R: ATGAGAGATTCAGCCCAC
Lipin 1 *(LPIN1)*	F: ATGAGGACACAGCACTGA R: TTAGGAATATCATCTTGGAATGGT
Fatty acid translocase – *(CD36)*	F: TTACACATACAGAGTTCGTTA R: AGTGAAGGCTCAAAGATG
Adipose triglyceride lípase (*PNPLA2)*	F: ATTTCAGACAACTTGCCACTT R: AGATGTCACTCTCGCCTG
Stearoyl-Coenzyme A desaturase 1 *(scd1)*	F: GCTATCGGAATGTTAATGAT R: ATGGTTAATCCTGGCTAAT
Sterol Regulatory Element-Binding Protein 1 *(SREBF1)*	F:CGCTACCGTTCCTCTATCAATG R: TCAGCGTTTCTACCACTTCAG
Carbohydrate-responsive element-binding protein (*MLXIPL)*	F: CTTATGTTGGCAATGCTG R: GGCGATAATTGGTGAAGA
Diacylglycerol O-Acyltransferase *(DGAT 1)*	F: GACAGCGGTTTCAGCAATTAC R: GGGTCCTTCAGAAACAGAGAC
Interleukin 6 *(IL-6)*	F: CTGGAGTTCCGTTTCTACCTG R: CCTTCTGTGACTCTAACTTCTCC
Tumor necrosis factor *(TNF- α)*	F: CTTCTCATTCCTGCTCGTGG R: TGATCTGAGTGTGAGGGTCTG
Adiponectin (*ADIPOQ)*	F: AAGTCTGGCTCCAAGTGTATG R: AGCAATACAATCAACCTCTCAAAC
Forkhead box o1 (FOXO1)	F: GGATAAGGGCGACAGCAACA R: TGAGCATCCACCAAGAACT
Peroxisome proliferator-activated receptor coactivator 1 (*PPARGC1A*)	F: TGTTCCCGATCACCATATTCC R: CTTCATAGCTGTCATACCTGGG

Table 2.1 – Forward and Reverse primers sequences used for RT-PCR (cont.)

Primer	5' – 3' sequence (foward; reverse)
Protein tyrosine phosphatase 1b (*PTPN1*)	F: CCTATTCAAAGTCCGAGAGTC R: GCATCTCCAACAGCACTTTC
Glucose-6-phosphatase *(G6PC)*	F: GTGAATTACGAAGACTCCCAGG R:TGTTTTATCAGAGGCAVGGAG
Phosphoenolpyruvate carboxykinase (*Pck2*)	F: ATCACCAACCCCGCAGGGAAAA R:TTGGATGCGCACAGGGTTCCTT
Glucokinase *(GCK)*	F:TCAGGAGTCAGGAACATC R: TTGTAGTATCCATAGCCATCT
Insulin receptor (Insr)	F:CACAACCTCACGATCACTCAG R: AGAACAGCATGAATCCCAGG
Insulin receptor substrate 1 *(IRS1)*	F: ACGCTCCAGTGAGGATTTAAG R: CCTGGTTGTGAATCGTGAAAG
Glucose transporter 1 *(GLUT1)*	F: CCCACAGAGAAGGAACCAATC R: TGCAGTTCGGCTATAACACC
Glucose transporter 2 *(SLC2A2)*	F: AAGACAAGATCACCGGAACC R: GACAGAGACCAGAGCATA
Glucose transporter 4 *(GLUT4)*	F: CTTTAGACTCTTTCGGGCAGG R: CGTCATTGGCATTCTGGTTG
Glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)*	F: AACGACCCCTTCATTGACC R: CACGACATACTCAGCACCAG
TATA –binding protein *(TBP)*	F: ACCCCACAACTCTTCCATTC R: CAAGTTTACAGCCAAGATTCACG

2.7 Protein extraction

Perirenal and epididymal adipose tissue (200mg), liver (25 mg) and muscle (50 mg) were cut and placed in ice-cold buffer, 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, 10 µg/ml Aprotinin from bovine lung, 1 mM Benzamidine and 1 mM Phenylmethylsulfonyl fluoride (PMSF), (Sigma-Aldrich, St. Louise, MO, USA). Samples were then homogenised and lysed at 4ºC in Tissue Lyser. The insoluble substances were sedimented through centrifugation (14000RPM for 10 min) and stored at -80ºC.

2.7.1 Immunoblotting

Protein concentration was determined using the bicinchoninic acid method (Pierce® BCA, Thermo Scientific, Rockford, IL, USA). and lysates were denatured at 95ºC, for 5 min, in sample buffer (0.125 mM Tris pH 6.8; 2% w/v SDS; 100 mM DTT; 10% glycerol and bromophenol blue) for its use in western blot analysis. Equal amount of protein from

each sample were loaded in polyacrylamide gels and proteins were separated according to their electrophoretic mobility using SDS-PAGE. Proteins were then transferred to a PVDF membrane. The membranes were blocked with 5% (w/v) fat-free dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T), for 1 h, at room temperature. After washing, membranes were incubated overnight at 4ºC with the primary antibodies against the different proteins of interest (Table 2.2). After incubation, membranes were washed and incubated for 1 h at room temperature, with alkaline phosphatase-conjugated anti-rabbit antibody (1:5000), anti-mouse antibody (1:5000) or anti-goat antibody (1:5000) (Santa Cruz Biotechnology, Inc, CA, USA). The membranes were exposed to ECF reagent followed by scanning for blue excited fluorescence on the VersaDoc (Bio-Rad Laboratories, Amadora, Portugal). The generated signals were analyzed using the Image-Quant TL software.

Table 2.2 - List of antibodies used for Western blot, source and dilution.

Antibody	Dilution	Company
SREBP	1:1000	Santa Cruz Biotechnology
ChREBP	1:500	Santa Cruz Biotechnology
ACC1	1:1000	Cell Signaling Technologies
FAS	1:1000	Cell Signaling Technologies
HSL	1:1000	Abcam Inc.
DGAT1	1:200	Santa Cruz Biotechnology
G6Pase	1:2000	Santa Cruz Biotechnology
PEPCK	1:2000	Santa Cruz Biotechnology
GK	1:1000	Santa Cruz Biotechnology
FOXO1	1:1000	Cell Signaling Technologies
PGC1-α	1:1000	Santa Cruz Biotechnology
IR	1:1000	Santa Cruz Biotechnology
IR Tyr1146	1:500	Cell Signaling Technologies
IRS-1	1:1000	Cell Signaling Technologies
IRS-1 Tyr612	1:500	Invitrogen
PI3Kp85	1:500	Cell Signaling Technologies
Akt Ser473	1:1000	Cell Signaling Technologies
Akt Thr308	1:500	Santa Cruz Biotechnology
Akt	1:500	Cell Signaling Technologies
Akt 2/β	1:1000	Millipore

Table 2.2 - List of antibodies used for Western blot, source and dilution (Cont.)

Antibody	Dilution	Company
AS160 Ser642	1:500	Santa Cruz Biotechnology
AS160	1:500	Millipore
p70S6K Thr421/424	1:1000	Cell Signaling Technologies
P70S6K	1:1000	Cell Signaling Technologies
GLUT1	1:1000	Millipore
GLUT2	1:2000	Millipore
GLUT4	1:500	Millipore
mTOR Ser2448	1:500	Millipore
mTOR	1:1000	Santa Cruz Biotechnology
β-actin	1:5000	Cell Signaling Technologies
α-Tubulin	1:5000	Cell Signaling Technologies

2.8 - $^{2}H_2O$ enrichment

A loading dose of 99% $^{2}H_2O$ in saline was administrated by injection to the intraperitoneal cavity in all animals 72 h before the sacrifice. Assuming 70% body weight as water, the dose was designed to aim at 5% body water enrichment. After the bolus injection, the drinking water was kept at 5% $^{2}H_2O$ enrichment to ensure steady body water enrichment until the end of the experiment. The animals were fasted overnight and on the day of sacrifice, the animals were given intraperitoneal injection of non-ionic detergent Pluronic F-124 (1000 mg/kg). In order to estimate hepatic VLDL production blood samples were collected before, and 2 and 4 h after the detergent injection.

2.9 - Metabolite preparation

2.9.1 - Hepatic glycogen extraction.

Glycogen was extracted from frozen liver powder by treatment with 30% KOH (2 ml/g of liver) at 70ºC for 30 minutes. After vigorous vortex, the mixture was treated with 6% Na_2SO_4 (1 ml/g of liver) and 99.9% ethanol (to a final concentration of 70%, 7 ml/g of liver) and left overnight at 4ºC to precipitate glycogen. After centrifugation, the upper liquid phase was discarded and the solid residue dried. The residue was resuspended in acetate buffer (0.05 M, pH = 4.5), and 20 µL of an aqueous solution containing 16 U of

amyloglucosidase from *Aspergillus niger* (Glucose-free preparation, Sigma-Aldrich, Germany) was added. Samples were incubated overnight at 55ºC and centrifuged. The remainder was lyophilized.

2.9.2 - Derivatization of glucose to monoacetone glucose (MAG).

In order to optimize the signal resolution in the NMR spectra, glucose obtained from glycogen isolation and hydrolysis was derivatized to MAG. Glucose was vigorously mixed with 5 mL acetone containing 4% sulphuric acid (v/v), both enriched with deuterium to 2%. The mixture was stirred overnight at room temperature to yield diacetone glucose. The acetonation reaction was quenched by adding 5 mL of water (also enriched with deuterium to 2%) and the pH was adjusted to pH 2.0 with 1M HCl. The newly formed diacetone glucose was hydrolyzed to MAG by incubation at 40ºC for 5 hours. The solution pH was then increased again to 8 with 1M $NaHCO_3$ and the samples were dried by rotary evaporation under vacuum. MAG in the residue was extracted with boiling ethyl acetate. Following evaporation of ethyl acetate, the residue was dissolved in H_2O and purified by solid phase extraction on reverse phase prepacked columns (Sigma prepacked 0.5g Discovery DSC-18 SPE Tubes).

2.9.3- Hepatic lipid extraction and purification.

Hepatic lipids were extracted from freeze-clamped livers by a Folch extraction. To a macerated liver 20 ml of chloroform/methanol mixture (2:1, v/v) per gram of liver was added and the mixture stirred at room temperature for 1 h. After centrifugation, collected supernatant was washed with 0.9% NaCl solution (4 ml/ gram of liver). After the layers were separated by centrifugation, organic layer was collected and evaporated to dryness. Triglyceride fraction was separated from the rest of the lipids by column chromatography according to the modified procedure described previously (Hamilton *et al.*, 1988). Lipid mixture was dissolved in hexane/methyl-*t*-buthyl-ether (MTBE) mixture (200:3, v/v) and applied to a silica gel prepacked column (Sigma prepacked 2g Discovery DSC-Si SPE Tubes). The column was eluted with the hexane/MTBE mixture (200:3, v/v) and the fractions containing triglycerides evaporated to dryness. To determine the identity and the purity of collected lipid fractions, TLC was carried out on silica gel plates. Petrolether/diethylether/acetic acid (7:1:0.1) system was used as mobile phase (triglyceride R_f = 0.55). The lipid fractions were visualized by iodine vapours.

2.9.4 - NMR analysis

Plasma body water ^{2}H-enrichments were determined from 10 µL of plasma by ^{2}H NMR as described previously (Jones *et al.*, 2001). NMR spectra of triglyceride and MAG samples were obtained with a 14.1 T with Agilent 600 system equipped with a 3-mm broadband probe. Triglyceride samples were dissolved in chloroform and the internal pyrazine standard was added. Fully relaxed ^{1}H and ^{2}H spectra of triglycerides were acquired at 25 ºC with a 90º pulse and 8 s of recycling time (3 s of acquisition time and 5 s pulse delay). MAG samples were dissolved in 90% acetonitrile / 10% water. ^{2}H MAG spectra were acquired at 50 ºC using a 90º pulse and 1.7 s of recycling time (1.6 s of acquisition time and 0.1 s pulse delay). The summed free induction decays were processed with 1.0 Hz line-broadening before Fourier transform. Spectra were analyzed with the NUTS PC-based NMR spectral analysis software (Acorn NMR Inc., USA).

2.9.5 - Quantification of direct and indirect pathway contributions to hepatic glycogen production.

MAG ^{2}H positional enrichments were estimated from the ^{2}H peak intensities of individual positions compared to the enrichment of methyl groups of isopropylidene moiety [Eq.1]

[Eq.1] $^{2}H_i$ (%) = [area $^{2}H_i$ x acetone (%)] / Δ area ^{2}H CH_3,

($^{2}H_i$ (%) = the enrichment of individual MAG position, area $^{2}H_i$ the area of individual peak, Δ area ^{2}H CH_3 the area of CH_3 isopropylidene peaks, and acetone (%) acetone enrichment).

The fractional contribution of the indirect pathway to hepatic glycogen production was calculated from the ratio of ^{2}H enrichments in positions 5 and 2 of hepatic MAG, as previously reported (Jones *et al.*, 2001) [Eq.2]:

[Eq.2] Indirect pathway (%) = 100 x $^{2}H_5$ (%) / $^{2}H_2$ (%)

($^{2}H_5$ (%) and $^{2}H_2$ (%) are the enrichments of MAG positions 5 and 2, respectively)

The contribution of the direct pathway to hepatic glycogen production was obtained from [Eq.3]:

[Eq.3] Direct pathway (%) = 100 x {1 - [$^{2}H_5$ (%) / $^{2}H_2$ (%)].

The fraction of glycogen turnover during D_2O exposure or the total hepatic glycogen that was synthesized by both pathways was calculated as the ratio of ^{2}H enrichment in MAG position 2 and body water enrichment [Eq.4]:

[Eq.4] Glycogen turnover (%) = 100 x $^{2}H_2$ (%) / BW (%).

2.9.6 - Quantification of the fractional de novo lipogenesis and glyceroneogenesis.

Triglyceride ^{2}H-enrichments were quantified from the ^{1}H and ^{2}H NMR spectra by measuring the ^{1}H and ^{2}H intensities of selected signals relative to the ^{1}H and ^{2}H intensities of the pyrazole standard.

The fraction of hepatic triglyceride derived from DNL over the duration of $^{2}H_2O$ administration was calculated as follows [Eq.5]

[Eq.5] DNL fraction (%) = 100 × TG-methyl (%) / BW (%)

(TG-methyl (%) is the ^{2}H enrichment of the triglyceride methyl groups and BW (%) the ^{2}H enrichment of the body water)

The fraction of *de novo* synthesized glycerol was calculated in a similar fashion from the ratio of glycerol positions 1 and 3 (G1,3) ^{2}H enrichment to that of ^{2}H body water enrichment [Eq.6]:

[Eq.6] Glyceroneogenesis fraction (%) = 100 × G1,3 (%) / BW (%)

2.10 Statistical analysis

Results are given as mean ± standard error of the mean unless stated otherwise. A p-value <0.05 was considered statistically significant. The differences between groups were tested by performing analysis of variance (ANOVA), followed by the Bonferroni Post'hoc test. The unpaired t-test was used for statistical analysis of differences between the same treatments at different time point. Statistical analyses were performed using the GraphPad Prism software, version 5 (GraphPad Software Inc., La Jolla, CA, USA).

2.11 Chemicals

Cyclosporine A (Sandimmune Neoral®) was kindly supplied by Novartis Pharma (Lisbon, Portugal), while SRL (Rapamune) was provided by Wyeth Europe Ltd (Berkshire, UK). Human insulin, Actrapid, 100 U/ml was a kind gift from Novo Nordisk A/S (Paço de Arcos, Portugal). Collagenase, type II from *Clostridium histolyticum,* and glucose strips were purchased from Roche (Lisbon, Portugal). D- [U-14C] glucose (specific activity, 200-300 mCi/mM) was purchased from Scopus Research BV (Wageningen, The Netherlands). Diethyl pyrocarbonate (DEPC) was acquired from AppliChem, (Darmstadt, Germany). Methanol and isopropanol were obtained from Merck (Darmstadt and ohenbrunn rispectively, Germany). All primers were designed by us through the VECTOR NTI Advanced 10 software (Life Technologies, Carlsbad, CA, USA) and obtained from IDT-Integrated DNA Technologies, Inc (Coralville, IA, 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). The enhanced chemifluorescence (ECF) reagent was obtained from GE Healthcare (Fairfield, CT, USA). All other reagents were purchased from Sigma Chemical Co.

Chapter 3

Effects of Cyclosporine A and Sirolimus on insulin-stimulated glucose transport and glucose tolerance in a rat model

This Chapter comprises the work published in
Transplantation proceedings (2013) by
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Reis F, Carvalho, E

3.1 Introduction

The introduction of calcineurin inhibitors, like cyclosporine A (CsA), has been important to save lives and improve the safety of organ transplantation. However, the use of these drugs is followed by the emergence of a number of side effects that has an outcome in the patient´s quality of live. One of the most important is new-onset diabetes mellitus after transplantation (NODAT) (Heisel *et al.*, 2004; Øzbay *et al.*, 2012; Vincenti *et al.*, 2007), which is usually associated with an increased risk of cardiovascular diseases and consequently decreased patient survival (Cosio *et al.*, 2005 ; Cosio *et al.*, 2002 ; Øzbay *et al.*, 2012).

CsA, a peptide of fungal origin, forms a complex with cyclophilins, which then inhibits calcineurin preventing, the movement of transcription factors into the nucleus, thus blocking interleukin (IL)-2 production and, consequently, proliferation and differentiation of T-cells (Sarwal *et al.*, 2001; Smith *et al.*, 2003). Studies on purified islets and insulin-producing beta-cells have proposed different diabetogenic actions for CsA. Therefore, CsA decreases insulin content of the β-cell, reversibly inhibiting insulin gene transcription and ultimately insulin secretion (Øzbay *et al.*, 2011) although the mechanisms that lead to these effects are not well understood.

Sirolimus (SRL), a macrolide of anti-fungal origin, inhibits the mammalian target of rapamycin (mTOR), is a recent option for immunosuppressive therapy (Mota, 2005). mTOR exists in two physically and functionally distinct multi protein complexes located in the cytoplasm and in the nucleus; the mTOR complex 1 (mTOR, raptor, mLTS8, deptor and the regulatory component PRAS40) and the mTOR complex 2 (mTOR, rictor, stress-activated protein kinase interacting protein 1, protor, mLST8 and deptor) (Dowling *et al.*, 2010; Pereira *et al.*, 2012; Rosner *et al.*, 2008). SRL has been viewed as an alternative to calcineurin inhibitors as its immunosuppressive activity has been reported to be up to 100 times greater than CsA (Gueguen *et al.*, 2007; Kay *et al.*, 1989). It is associated with several side effects, including proteinuria, diabetes and impaired wound healing (Cravedi *et al.*, 2010; Cutler *et al.*, 1999 ; Pereira *et al.*, 2012). Another well-known side effects of SRL and CsA is hyperlipidemia, which is usually associated with increased serum cholesterol and triglycerides (TG) values during the first year of treatment (Badiou *et al.*, 2009; Gueguen *et al.*, 2007). Nonetheless, SRL is putatively less nephrotoxic than CsA, an advantage that could be lost due to the deleterious effects on glucose and lipid metabolism, which would be particularly problematic in diabetic and dyslipidaemic patients. As long as the precise molecular mechanisms underlying the effects of SRL and CsA on glucose and lipid metabolism remain to be elucidated, the most proper immunosuppressive protocols for each specific condition are as yet unknown. Long-term outcomes may not improve significantly. The clinical follow-up data are yet insufficient; animal studies focusing on clinically relevant doses of the drugs are advised. Thus, the aim of this study was to evaluate and compare the potential metabolic effects of either CsA or SRL *ex vivo* treatments of isolated rat adipocytes and further to evaluate the long-term *in vivo* treatment effects of CsA or SRL, on glucose and lipid metabolism in Wistar rats.

3.2 Results

3.2.1 Effects of CsA and SRL on glucose uptake in isolated rat adipocyte - ex vivo

To evaluate the effect of CsA and SRL on glucose uptake, freshly isolated adipocytes were incubated *ex vivo* with different concentrations of these drugs in the presence or absence of 10 nM insulin for 10 min. As indicated in Figure 3.1 A and B, incubation of isolated adipocytes with a maximal insulin concentration resulted in a 7- to 8- fold increase over basal in the insulin-induced glucose uptake rate. In contrast, cells incubated with varying concentrations of CsA (0.5 –30 µM) in the presence of 10 nM insulin, showed significant inhibition of the insulin-stimulated glucose uptake (Fig. 3.1A). For the lowest CsA concentration used (0.5 µM), we already observed a 39% ($p<0.05$) reduction in the insulin-stimulated glucose uptake and for 30 µM, 41% ($p<0.05$). Adipocytes incubated with SRL (5-250 µM) in the presence of 10 nM insulin, displayed inhibition of the insulin-stimulated glucose uptake, which was nearly significant at 5 µM ($p<0.06$) and was significant at 250 µM incubation (77 % reduction, $p<0.05$) (Fig. 3.1B).

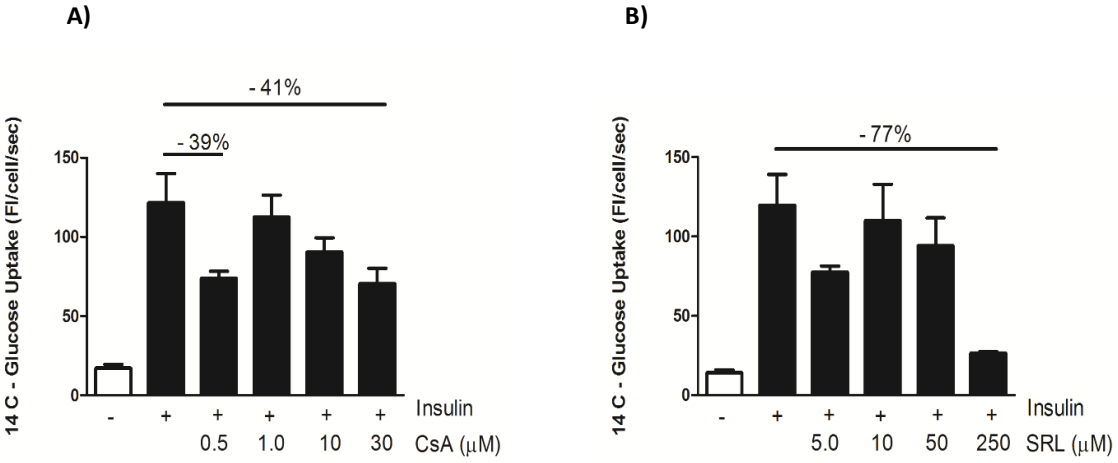


Figure 3.1 – Effects of CsA and SRL on glucose uptake in epididymal adipocytes. D-[U-14C]-glucose uptake was measured in isolated rat adipocytes, incubated *ex vivo* for 30 minutes with either (A) CsA: 0.5-30 µM, (n=6), (B) SRL: 5-250 µM, (n=9) either in the presence or absence of 10 nM Insulin, before D-[U-14C]-glucose was added, and glucose uptake was assessed during the following 30 min. Data are presented as mean ± SEM. Differences between treatments were assessed with one-way ANOVA.

3.2.2 Effects of CsA and SRL on glucose uptake in isolated rat adipocytes - in vivo

After 3 and 9 weeks of CsA and SRL treatments, the insulin-stimulated glucose uptake response was measured in freshly isolated rat adipocytes. After 3 weeks, the vehicle group, showed about a 3-fold increase in insulin-stimulated glucose uptake compared to basal (Fig 3.2A), whereas the CsA-treated group displayed a significantly blunted response to insulin (47%) compared with the vehicle cohort. In addition, we also observed a significant reduction of 27% in glucose uptake in response to insulin in the SRL group compared with vehicle (Fig 3.2A). Furthermore, after 9 weeks of treatments, insulin stimulated glucose uptake in isolated adipocytes of vehicle-treated animals remained at about 3-fold over basal, whereas CsA treatment produced a smaller but significant decrease in insulin-stimulated glucose uptake (9%) compared with vehicle (Fig 2.2B). Moreover, after 9 weeks, the SRL-treated group showed a 24% reduction in insulin-stimulated glucose uptake compared with vehicle (Fig 3.2B).

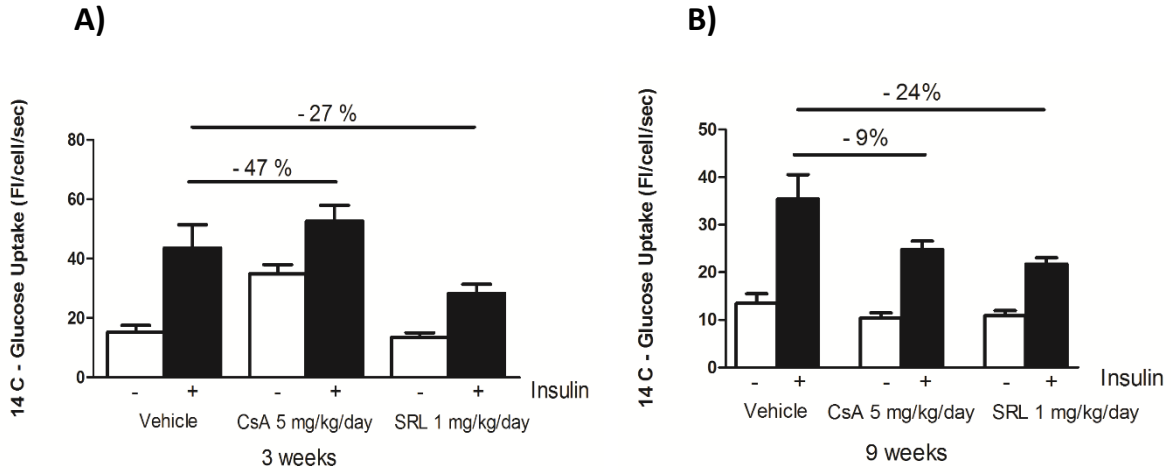


Figure 3.2 – Effects of CsA and SRL on glucose uptake in epididymal adipocytes. D-[U-14C]-glucose uptake after *in vivo* treatment with either CsA (5 mg/kg/day) or SRL (1 mg/kg/day) for either 3 (A) or 9 weeks (B). Glucose uptake was measured in, freshly-isolated epididymal adipocytes, that were pre-incubated during 10 min with or without insulin (10 nM), before D-[U-14C]-glucose was added, and glucose uptake was assessed during the following 30 min. Data are presented as mean ± SEM. Differences between treatments were assessed with one-way ANOVA.

3.2.3 Drug Blood Concentration

The trough blood concentration of CsA and SRL obtained using the doses of 5 mg/kg/day and 1 mg/kg/day, respectively, was 367 ± 45.5 ng/ml and 7.8 ± 1.9 ng/ml, respectively.

3.2.4 Effects of CsA and SRL on body weight

Alterations in body weight were monitored during *in vivo* treatment (Fig. 3.3). Wistar rats had a mean weight of 310 ± 2.1 g at 10 weeks of age before the beginning of treatments. As observed in Fig 3.3, neither treatment for 3 weeks caused significant changes in body weight gain compared with vehicle. After 9 weeks, however, both vehicle-treated (412 ± 10.1 g) and CsA-treated (420.2 ± 6.9 g) animals showed similar body weight, whereas SRL treatment caused a smaller body weight gain, already significant compared with CsA-treated animals after 5 weeks (352.8 ± 7.0 vs. 388 ± 5.4 g; $p < 0.01$). These observations were maintained until the end of treatments, when the SRL group reached a significant difference from vehicle (380.2 ± 8.0 vs. 412 ± 10.8 g; $p<0.05$) (Fig 3.3).

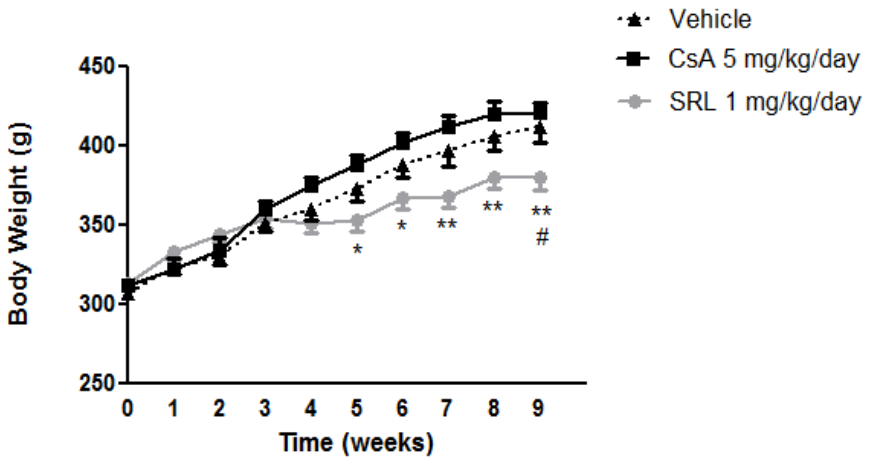


Figure 3.3. - Effects of vehicle, CsA and SRL on body weight. Weight was monitored every week until the end of treatments to evaluate the effects of CsA (5 mg/kg/day) and SRL (1 mg/kg/day). Data are presented as mean ± SEM. Differences between treatments were assessed with one-way ANOVA. $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0,001$ vs. vehicle; * $p<0.05$, ** $p<0.01$, *** $p<0,001$ SRL vs. CsA at the same time point.

3.2.5. Effects of CsA and SRL on epididymal fat pad weight and adipocyte size

After sacrifice, epididymal fat pads were weighted to calculate fat pad weight/BW (Fig 3.4). The SRL-treated group showed a significant decrease in fat pad weight (0.013 ± 0.0 vs. 0.018 ± 0.0 g; $p<0.01$) compared with either vehicle or CsA-treated animals (0.013 ± 0.0 vs. 0.019 ± 0.0 g; $p<0.01$) after 3 weeks, although there were no significant changes in fat pad weights after 9 weeks.

In addition, CsA treatment after 9 weeks significantly increased fat cell diameter (106.9 ± 4.5 vs. 83.9 ± 1.7 µm; $p<0.01$) and weight (0.59 ± 0.08 vs. 0.35 ± 0.04 µg; $p<0.05$)

compared with vehicle and with SRL cell diameter (106.9 ± 4.5 vs. 85.9 ± 2.6 µm; p<0.01) and weight (0.59 ± 0.08 vs. 0.32 ± 0.02 µg; p<0.01) (Table 3.1).

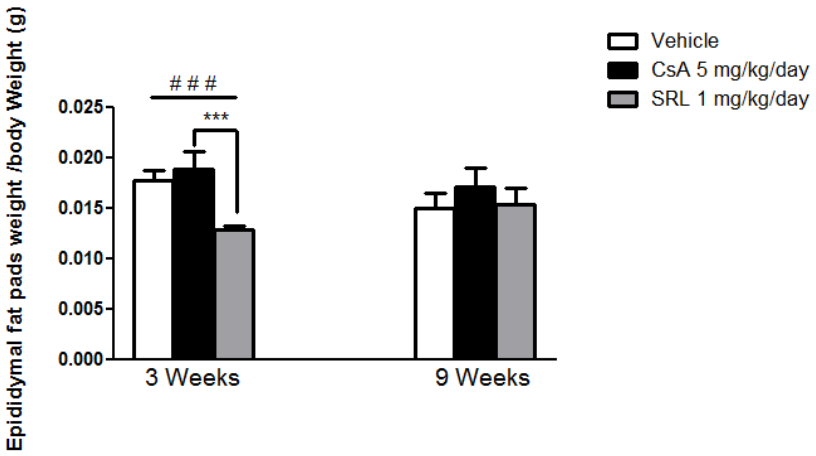


Figure 3.4. - Effects of vehicle, CsA and SRL fat pad weight. Epididymal fat pads were harvested and weighted after sacrifice at 3 and 9 weeks to evaluate the effects of CsA (5 mg/kg/day) and SRL (1 mg/kg/day). Data are presented as mean ± SEM. Differences between treatments were assessed with one-way ANOVA. ### p<0.001 vehicle vs. SRL group; *** p<0.001 CsA vs SRL group.

3.2.6. Effects of CsA and SRL on glucose tolerance and insulin levels

Fasting serum glucose values were not different between the groups after 3 weeks but were significantly different after 9 weeks of treatments between CsA (101.5 ± 3.2) and vehicle (95.6 ± 3.1, p<0.01) and SRL (87.1 ± 3.3 mg/dl; p< 0.01; Table 1). Fasting serum insulin levels between CsA and SRL-treated animals were also not different after 3 weeks, but were significantly different after 9 weeks (58.5 ± 4.5 vs. 134.5 ± 2.2 pmol/L; p<0.05). Moreover, HOMA-IR was also calculated based on the fasted glucose and insulin values. The SRL group presented a higher value for this index, 2.02 after 3 weeks and 3.08 after 9 weeks compared to the other groups (Table 3.1). In addition, fed glucose levels were similar after 3 weeks, between treatments (Table 3.1). Curiously, fed glucose for vehicle-treated animals after 9 weeks was lower compared to 3 weeks (157.5 ± 7.4 vs. 219.5 ± 11.2; p<0.05). These values was increased significantly among the CsA-treated group (237 ± 27.8 mg/dl) compared with the vehicle-treated group (157.5 ± 7.4 mg/dl, p<0.05). In addition, glucose levels were not different in the SRL-treated group (Table 3.1). Fed insulin levels revealed no difference between treatments after 3 weeks. The SRL-treated group, displayed an

increase in fed insulin between 3 and 9 weeks (170.7 ± 12 vs. 212.1 ± 10, $p<0.05$) and a significant increase compared with CsA after 9 weeks (212.1 ± 10 vs. 158.4 ± 9.7, $p<0.01$).

3.2.7 Effects of CsA and SRL on serum lipid

Serum TG in fed state were not different among treatments after 3 weeks, but were increased for vehicle and CsA group after 9 weeks (Table 3.1). TG were significantly increased in the vehicle group after 9 weeks as compared with 3 weeks (88 ± 9.5 vs. 118.4 ± 9.1 mg/dl; $p<0.05$). In addition, TG values were also significantly increased after 9 weeks with CsA (184.7 ± 14.6 vs. 93.5 ± 9.2 mg/dl; $p<0,001$) compared with 3 weeks. No significant differences were observed at 3 versus 9 weeks in the SRL-treated group. After 9 weeks, TGs were also significantly increased in the CsA group (184.7 ± 14.6 vs. 118.4 ± 9.1 mg/dl; $p< 0.001$) compared with vehicle and with SRL-treated group (184.7 ± 14.6 vs. 109.1 ± 11.3 mg/dl; $p<0.001$). Moreover, serum total cholesterol levels in the fed state were significantly different after 3 weeks of treatment between vehicle (52.5 ± 2.4) and the SRL-treated group (64.9 ± 3.2 mg/dl; $p<0.01$). In addition, after 9 weeks of SRL treatment total cholesterol levels were different (51.7 ± 1.5 vs 60.1 ± 3.7; $p<0.05$) compared with vehicle. No significant differences were observed with the CsA treatment (Table 3.1).

Table 3.1. Acute (3 weeks) and chronic (9 weeks) effects of CsA and SRL

	3 Weeks			9 weeks		
	Vehicle	Cyclosporin A	Sirolimus	Vehicle	Cyclosporin A	Sirolimus
BW (g)	350.30 ± 4.98	359.80 ± 5.47	353.21 ± 5.88*	412.60 ± 10.09**	420.18 ± 6.89***	380.17 ± 8.05*
Fat Cell diameter (µm)	75.08 ± 3.25	78.72 ± 2.45***	72.70 ± 2.20***	83.94 ± 1.67*	106.88 ± 4.50***	85.92 ± 2.61$$$
Fat Cell Weight (µg)	0.27 ± 0.05	0.29 ± 0.02***	0.24 ± 0.02*	0.35 ± 0.04	0.59 ± 0.08**	0.32 ± 0.02$$
Fasted Glucose (mg/dl)	86.93 ±3.14	86.40 ± 2.93	86.93 ± 2.95	95.58 ± 3.09	101.50 ± 3.22**	87.08 ± 3.30$$
Fasted Insulin (pmol/l)	46.85 ± 6.33	53.55 ± 8.85	37.05 ± 5.70	94.55 ± 19.43	58.48 ± 4.47	134.50 ± 2.20$
HOMA-IR	1.56	1.95	2.02	2.67	2.47	3.09
Fed Glucose (mg/dl)	219.50 ± 11.21	218.50 ± 14.51	203.80 ± 7.61	157.50 ± 7.44*	237 ± 27.84*	208.30 ± 17.76
Fed Insulin (pmol/l)	152.80 ± 15.93	178.20 ± 13.99	170.70 ± 12.03*	178.30 ± 12.87	158.40 ± 9.71	212.10 ± 10.04$$
Fed Triglycerides (mg/dl)	88 ± 9.53*	93.50 ± 9.17	86.44 ± 6.37***	118.40 ± 9.11	184.70 ± 14.63***	109.10 ± 11.27$$$
Fed Cholesterol (mg/dl)	52.47 ± 2.42	55 ± 3.11	64.88 ± 3.18**	51.67 ± 1.50	54.20 ± 2.0	60.08 ± 3.72*

Data are means ± SEM. * p<0.05; ** p<0.01; *** p< 0.001 3 weeks vs 9 weeks in the same treatment group;
* p<0.05; ** p< 0.01; *** p<0.001 vs. vehicle at the same time point; $ p<0.05; $$ p< 0.01 $$$ p<0.001vs. CsA at the same time point

3.2.8 Effects of CsA and SRL on Glucose Tolerance Test

GTTs were performed both after 3 (Fig 3.5A) and 9 weeks (Fig 3.5B) of treatments. Fifteen minutes after a glucose bolus (2 g/kg BW, i.p.), the CsA-treated group presented significantly higher glucose levels (287.8 ± 31.9) when compared to either the vehicle (205.7 ± 24.2 mg/dl, $p<0.05$) or the SRL (213 ± 27.4; $p<0.05$) cohorts, after 3 weeks of treatment (Fig 3.5A). No significant differences were observed between SRL and vehicle-treated group after 3 weeks. Furthermore, after 9 weeks of treatments there were no significant differences in the fasted glucose values between the groups. However, with CsA treatment, glucose values were significantly increased compared with SRL at 15 minutes (472.6 ± 25.6 vs. 376.3 ± 29.9 mg/dl; $p<0.01$) and compared with vehicle at 30 minutes (436.8 ± 30.2 vs. 342.4 ± 16.5 mg/dl; $p<0.01$) and 60 minutes (295.7 ± 44.3 vs. 197.5 ± 13.5 mg/dl; $p<0.01$) compared to vehicle (Fig. 3.5B).

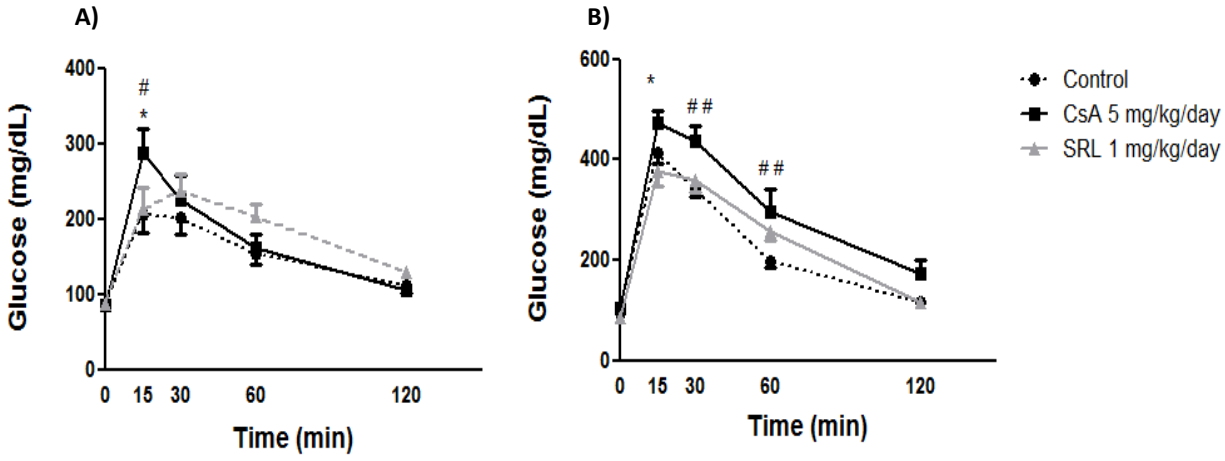


Figure 3.5. - Effects of vehicle, CsA and SRL on glucose tolerance tests after 3 weeks (A) and 9 weeks (B). Glucose levels at time point 0, and after an i.p. injection of glucose (2 mg/kgBW) at 15, 30, 60 and 120 min. Data are presented as mean ± SEM. Differences between treatments were assessed with one-way ANOVA. $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ vehicle vs. CsA group; * $p<0.05$ CsA vs SRL group.

3.3 Discussion

Using an animal model that mimics the doses used in clinical practice, CsA was shown to promote hyperglycemia, adipocyte insulin resistance, glucose intolerance, increased fat cell weight and hypertriglyceridemia. Moreover, CsA impairs insulin production/secretion in the fed state, which may contribute to the exacerbated glucose

intolerance. SRL also promoted hyperglycemia, adipocyte insulin resistance, preventing normal body weight gain.

Ex vivo short-term treatment of adipocytes with CsA and SRL caused concentration-depended inhibitory effects on the insulin-stimulated glucose uptake. A recent, *ex vivo* study using therapeutic concentrations of SRL added to human subcutaneous and omental adipocytes demonstrates inhibition of basal and insulin glucose uptake by these cells (Pereira *et al.*, 2012). When animals were treated with CsA or SRL, the insulin-induced glucose uptake, in isolated adipocytes, was impaired after 3 and after 9 weeks by both agents. In fact, a previous study *in vivo* study suggested that different doses and routes of administration of CsA reduces both active glucose uptake and passive fatty acid absorption by the intestine (Sigalet *et al.*, 1992). Our study suggested that these drugs taken either *ex vivo* or *in vivo* promote the same effect.

The doses of 5 mg/kg BW/day of CsA and 1 mg/kg BW/day of SRL in the *in vivo* study, achieved blood concentration mimicking those recommended during immunosuppressive therapy.

The 5 mg/kg/day dose of CsA promoted the same weight gain as the vehicle group, consistent with results by Böhmer *et al.* (2010) using the same dose for 8 weeks of treatment. In contrast, the SRL group (1 mg/kg/day) displayed a significantly less body weight gain after 5 weeks, similar to the results observed by Houde *et al.* (2010), who demonstrated reduced food intake, decreased food efficiency and increased energy expenditure. SRL inhibition of mTOR, which regulates cell proliferation and messenger RNA (mRNA) translation (Cutler *et al.*, 2001), may be one of the reasons for the smaller body weight gain. SRL treatment significantly reduced adipocyte cell diameter and weight, confirming observations by Chang *et al.* (2009). Furthermore, this was also reflected in the significantly decreased weight of the epididymal fat pads, compared with CsA or vehicle-treated animals, already after 3 weeks. However, the difference disappears after 9 weeks. These results seem contradictory to those in literature (Chang *et al.*, 2009; Houde *et al.*, 2010), but the discrepancy might be explained by our use of a smaller dose of SRL.

To assess glucose metabolism, fasted glucose and insulin levels were measured. Although after 3 weeks, no difference were seen in these two parameters, after 9 weeks treatment CsA induced higher fasting glucose levels with lower insulin levels than the other groups, reflecting a possible deficiency in insulin secretion/production. In contrast, SRL showed values of glucose similar to vehicle, but higher levels of insulin, suggesting insulin resistance. Furthermore, HOMA-IR indicated higher IR in older animals, including the

vehicle group (13 vs. 19 weeks old), although, the SRL-treated animals, showed a higher value of HOMA-IR, suggesting a greater degree of insulin resistance already after 3 weeks that worsened by 9 weeks of treatment. At the time of sacrifice, we assessed fed glucose and fed insulin values, which were similar between the groups after 3 weeks of treatments. However, after 9 weeks, glucose values were higher for rats treated with CsA, supporting the hyperglycemic effect of CsA. Similar results were observed in another study, in Sprague Dawley rats (Larsen *et al.*, 2006). Accordingly, insulin levels after 9 weeks decreased significantly in the CsA group, reflecting a defect in insulin secretion with a consequent increase in glucose levels in serum (Table 1).

Regarding the lipid profile, the CsA-treated group presented similar values of cholesterol as the vehicle, as observed also by Böhmer *et al.* (2010). In contrast, CsA given to rats for a longer time (Böhmer *et al.*, 2010; Vaziri *et al.*, 2000) increased TG, which may have been due to a significant decrease in plasma lipoprotein lipase activity (Espino *et al.*, 1995). A recent study suggested that both CsA and tacrolimus enhance lipolysis by regulating expression of critical lipogenic genes in human adipocytes. Increased values of lipids were expected in the SRL group because its hyperlipidemic characteristics are well known. Both serum cholesterol and TG are increased in humans during the first year of SRL therapy, after transplantation (Gueguen *et al.*, 2007; Kahan *et al.*, 1998; MacDonald *et al.*, 2000). In our study, TG values were similar to those among the vehicle even after 9 weeks of SRL treatment, but cholesterol was significantly increased in this group compared to vehicle after 3 and 9 weeks. Although surprisingly, this has also been observed in two recent studies with SRL (2 mg/kg/day), where plasma TG and NEFA levels of SRL-treated rats were similar to vehicle-treated rats (Chang *et al.*, 2009; Deblon *et al.*, 2012).

Moreover, during a GTT performed after 3 weeks, CsA cohort displayed significantly higher glucose levels after 15 minutes of the glucose bolus. Additionally, after 9 weeks, the CsA group, showed higher glucose levels at 15, 30 and 60 min with consequently a slower glucose excursion rate, reflecting a higher hyperglycemic/diabetogenic response. This observation could be the result of the inhibitory effects of CsA on β-cell survival therefore, causing the decrease in insulin secretion as observed by others (Øzbay *et al.*, 2012; Redmon *et al.*, 1996 ; Uchizono *et al.*, 2004). SRL may have decreased insulin sensitivity, which could explain the glucose excursion curve observed in the GTT after 3 weeks, although glucose levels were not as high as values observed at 15 minutes using CsA. At the later times during the GTT, however, the SRL glucose excursion curve did not return to basal levels as fast as the CsA and the vehicle

groups. After 9 weeks of SRL treatment, the glucose excursion curve was not different than vehicle. Houde *et al.* (2010) demonstrated that treatment of rats with SRL affects both glucose and insulin homeostasis, which led the authors to suggest that the insulin response during the GTT may be due to a possible defect in islet function.

In conclusion, this study demonstrated that both CsA and SRL promote hyperglycaemia, hyperlipidaemia and adipocyte insulin resistance. However, CsA is generally more diabetogenic than SRL, although, the SRL group displayed increased insulin levels reflecting insulin resistance, despite the observed reduction in body weight. These findings might be considered when choosing the proper immunosuppressive therapy for diabetic and/or obese individuals.

Chapter 4

Cyclosporine A enhances gluconeogenesis while sirolimus impairs insulin signaling in peripheral tissues after 3 weeks of treatment

This Chapter comprises the work submitted to

Biochemical Pharmacology (2014) by

Lopes PC, Fuhrmann A, Sereno J, Santos MR, Pereira MJ, Eriksson JW, Reis F, Carvalho E

4.1 Introduction

Immunosuppressive therapy is used in the treatment of autoimmune diseases and after organ transplantation, to promote tolerance to allografts (Smith *et al.*, 2003). Two of the main immunosuppressive agents are cyclosporine A (CsA), a calcineurin inhibitor and sirolimus (SRL), an mTOR inhibitor. Although these immunosuppressive agents are very effective in their function, they are also responsible for the development of metabolic complications, linked to higher rates of cardiovascular disease and infections, which is the major cause of morbidity and mortality after transplantation (Øzbay *et al.*, 2011; Subramanian *et al.*, 2007; Watt, 2011). One of the complications is NODAT, usually manifested in the first few months post-transplantation and varying according to the type of immunosuppressive agent, their different combinations and patient demographics (Dirks *et al.*, 2004). NODAT is reported in 2.5 to 40% of patients that underwent renal, liver, heart or lung transplant (Pham *et al.*, 2011). Similar to type 2 diabetes, NODAT has been associated with impairment in glucose tolerance and insulin secretion and dysfunctional hepatic gluconeogenesis (Hecking *et al.*, 2013). Insulin directly regulates gluconeogenesis, however in insulin resistance states insulin does not properly suppresses gluconeogenesis in the liver, leading to enhanced activation of forkhead box-containing transcription factors of the FOXO subfamily, promoting increased transcription of glucose-6-phosphatase (G6Pase) and

phosphoenolpyruvate carboxykinase (PEPCK), rate-limiting enzymes in hepatic glycogenolysis and gluconeogenesis, respectively (Nakae *et al.*, 2002; Pajvani *et al.*, 2011). Moreover, Ropelle *et al*. (Ropelle *et al.*, 2009) refer that the physical interaction of peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1α) and FOXO1 promote an important signal transduction pathway responsible for the synthesis of glucose by the liver. Furthermore, PGC-1α expression is a tissue-specific regulatory markers activated in diabetic states, as well as in the fasted state and perhaps responsible for increased hepatic glucose production and consequently hyperglycemia (Herzig *et al.*, 2001; Ropelle *et al.*, 2009), making it a marker of interest together with its downstream targets.

On the other hand, insulin is an intervenient in many physiological processes, particularly important in maintaining glucose homeostasis. After a meal, glucose increases in circulation, stimulating the secretion of C-peptide and insulin, which inhibit glycogenolysis and gluconeogenesis and at the same time promoting glycogen synthesis and glucose uptake. Insulin binds to its cell surface receptor (IR), activating its intrinsic tyrosine kinase and leading to receptor auto-phosphorylation, which in turn leads to the phosphorylation of insulin receptor substrates proteins (IRS-1 – IRS-4). As a result, several downstream signaling pathways are activated, including p85 regulatory subunit of PI3-kinase and protein kinase B (Akt/PKB). This last step activates pyruvate dehydrogenase kinase 1 (PDK1) and Protein kinase C (PKC), leading to translocation of the glucose transporter (GLUT4) from intracellular vesicles to the plasma membrane (Rhodes *et al.*, 2002). Alterations in these signaling pathways may affect glycemia and lead to unwanted metabolic consequences like diabetes and dislipidemia (Rhodes *et al.*, 2002).Although CsA and SRL have been linked with NODAT, the underlying mechanisms are still not completely understood. SRL has been shown to improve insulin-stimulated glucose uptake and Akt/PKB phosphorylation in L-6 muscle cells, 3T3-L1 cells and in differentiated adipocytes (Berg *et al.*, 2002; Tremblay *et al.*, 2005; Tremblay *et al.*, 2001), while other studies have shown reduced glucose uptake (Lopes *et al.*, 2013a) and Akt/PKB phosphorylation in human mature adipocytes (Pereira et al, 2012). While calcineurin inhibitors have been involved in the inhibition of the phosphorylation of the IR, it has not been associated with alterations in expression or phosphorylation of proximal insulin signaling cascade proteins (Pereira *et al.*, unpublished; Shivaswamy *et al.*, 2013). Therefore, there is still a lack of consensus on regarding the underlying mechanism for NODAT caused by both CsA and SRL.

Recently we and other have reported that treatment with either CsA or and SRL lead to metabolic alterations in liver, muscle and adipose tissue possibly contribute to the

development of dyslipidemia and insulin resistance associated with immunosuppressive therapy however no insulin signaling studies had been performed (Böhmer *et al.*, 2010; Fuhrmann *et al.*, 2014; Lopes *et al.*, 2013a; Øzbay *et al.*, 2011; Pereira *et al.*, 2012; Shivaswamy *et al.*, 2010),. Therefore, the main aim of this in vivo study is to understand how these immunosuppressive agents affect gluconeogenesis and insulin signaling in liver, muscle and adipose tissue after 3 weeks of treatment in a rodent model.

4.2 –Results

4.2.1 GTT, ITT as well as glucose, insulin and C-peptide measurements in serum

GTTs were performed at the end of the treatments and revealed that glucose tolerance was impaired in the CsA-treated animals. The CsA-treated group displayed a peak of glucose (18.62 ± 1.80 mmol/l) 15 minutes after the glucose bolus (2 g/kg BW, i.p.), when compared to either the vehicle group (11.16 ± 1.56 mmol/l, $p<0.001$) or the SRL-treated group (10.97 ± 1.68 mmol/l; $p<0.001$) (Fig 4.1A). However no significant differences were observed in the remaining time points during the GTT between the vehicle and the SRL group. The glucose excursion curve for SRL was also impaired and the recovery kinetics of blood glucose levels was significantly slower. Furthermore, the ITT curve revealed that insulin sensitivity was impaired in the CsA treated animals (Fig 4.1B). For the SRL group, a significant increase in blood glucose levels during an ITT was only observed at 60 min (3.76 ± 0.08 mmol/l) compared to vehicle group (2.65 ± 0.14 mmol/l, $p<0.05$). No significant differences were found in the glucose or insulin levels in the fasted state between groups, but a trend for a decrease in the fasting C-peptide levels was observed in both treated groups (Fig. 4.1C, D and E).

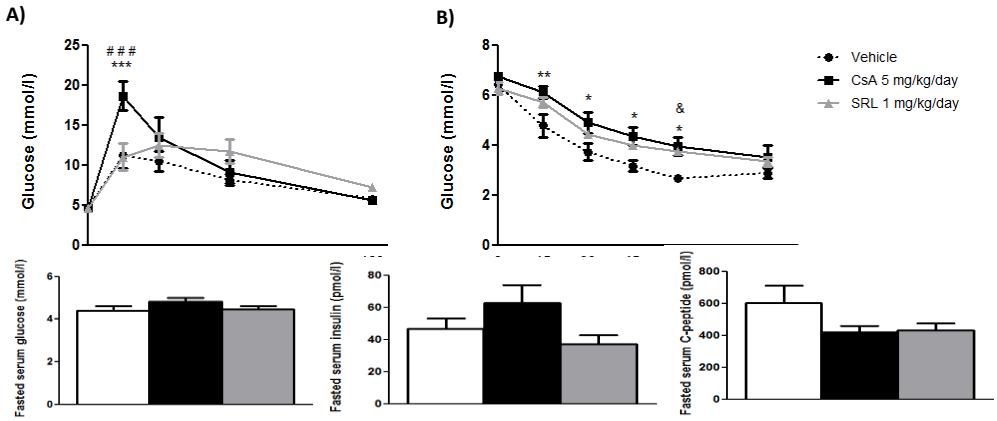


Figure 4.1. Effects of vehicle, CsA, and SRL treatment during a GTT (A), an ITT (B) and fasted serum glucose (C), insulin (D) C-peptide (E). Rats were treated with CsA and SRL for 3 weeks and fasted for 16 hours before the glucose tolerance test. Glucose levels were measured at time point 0, and after an intraperitoneal injection of glucose (2 g/kg BW) at 15, 30, 60, and 120 minutes. For the ITT, rats were fasted for 6 hours and glucose levels were measured at time point 0, and after an intraperitoneal injection of insulin (1U/kg BW) at 15, 30, 45, 60 and 90 minutes. Fasting serum glucose, insulin and C-peptide levels were measured. Data are presented as mean ± SEM (n=6-8/group) *p<0.05, ** p<0.01***p<0.001 vehicle vs. CsA group; $^{\&}$ p<0.05 vehicle vs. SRL group; $^{\#\#\#}$p<0.001 CsA vs. SRL group.

4.2.2 Clearance of glucose rate in the urine

Animals in the SRL group exhibited a trend to increase glucose clearance rate (0.14 ±0.04 ml/h/rat), compared with the CsA (0.06 ± 0.01 ml/h/rat) and the vehicle group (0.09 ± 0.01 ml/h/rat), which means that an excess of glucose is present in the urine flux and is being expelled via the kidneys (Fig. 4.2).

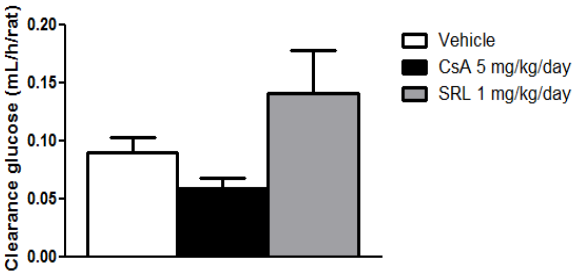


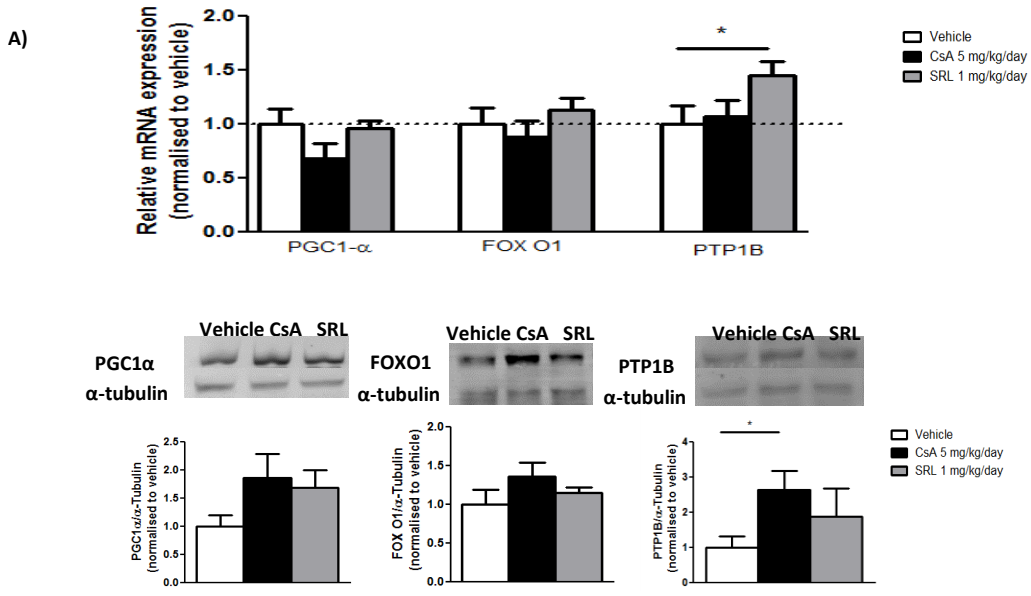
Figure 4.2. Effects of vehicle, CsA, and SRL treatment on glucose clearance rate in the urine. Rats were treated with CsA and SRL for 3 weeks and urine collection was done 24 h prior to sacrifice. Glucose levels was measured with strips (Cobas Integra® 400 plus). Data are presented as mean ± SEM (n=6-8/group).

4.2.3 Effect of CsA and SRL on protein and gene expression in liver

4.2.3.1 Gluconeogenesis is modulated by CsA and SRL

To evaluate if CsA or SRL treatment affect gluconeogenesis, we evaluated the liver expression levels of some of the important key players. Although no significant changes were observed in the transcription factors PGC1-α and FOXO1 at the gene level, a tendency for an increased in protein expression was observed in the CsA group (Fig. 4.3A). Moreover, a significant increase in protein expression for G6Pase was observed in the CsA group (68%, $p<0.05$) compared with vehicle group, while no changes were observed in gene level (Fig.4.3B). A trend for an increase in PEPCK both gene and protein expression was observed in the same group (Fig. 4.3B). Moreover, glucokinase protein, an important contributor to the formation of glycogen, as it is responsible for the phosphorylation of glucose into glucose-6-phosphate, was tendentiously decreased in the CsA group, being significantly reduced in the SRL group (35%, $p<0.05$) (Fig. 4.3B).

Moreover, we evaluated PTP1B expression, an important marker that negatively regulates insulin action, and found a significant increase in protein level in the CsA group (163%, $p< 0.05$) when compared to vehicle group (Fig. 4.3A).



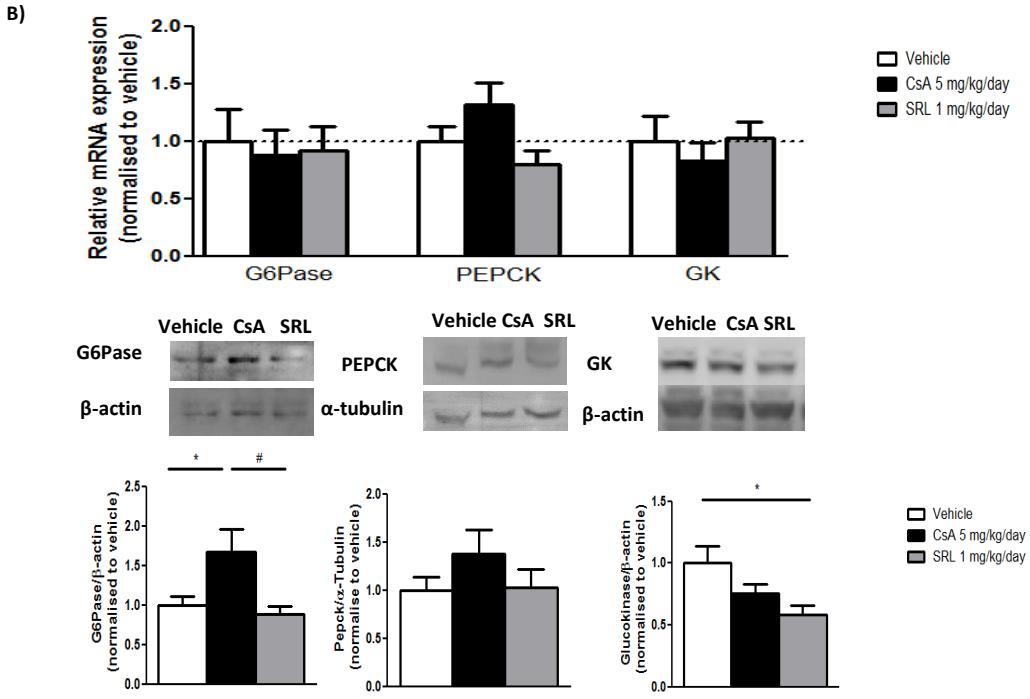


Figure 4.3. Gluconeogenic gene and protein expression in liver, after a 3 week-treatment period with CsA and SRL. Relative mRNA expression levels were determined by Real-time PCR (n=8) and protein expression levels determined by western blotting (three to five independent experiments) for PGC1-α, FOXO1 and PTP1B (A) and G6Pase, PEPCK and GK (B). Data are presented as mean ± SEM. *p<0.05 vehicle vs. CsA or SRL group; #p<0.05 CsA vs. SRL group.

4.2.3.2 Effect of CsA and SRL on insulin signaling in the liver

We also investigated the expression levels of important markers involved in insulin signaling in the liver. The IRS-1 protein level was increased in the SRL group (49%, p<0.05), while GLUT1 tended to be decreased in the same group (Fig. 4.4). No significant changes were observed in gene expression of IRS-1, GLUT1 and GLUT2 in both treated groups (Fig. 4.4).

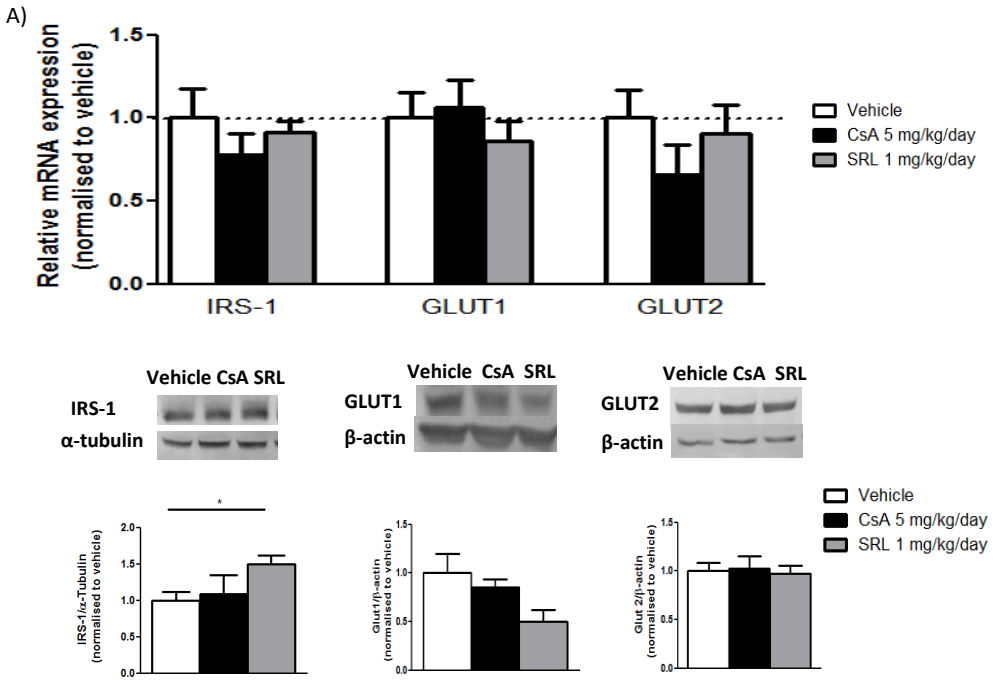


Figure 4.4. Expression of genes and proteins of the insulin signaling pathway in liver after a 3 week-treatment period, with CsA and SRL. Relative mRNA expression levels were determined by Real-time PCR (n=8) and protein expression levels determined by western blotting (three to five independent experiments) for IRS-1, GLUT1 and GLUT2. *p<0.05 vehicle vs. CsA or SRL group.

To further elucidate signaling events that might promote the impaired glycemia and glucose uptake seen in our previous work (Lopes *et al.*, 2013a), we studied the insulin cascade by western blot analysis in the group of animals treated with the insulin bolus (i.p 10 U/kg BW) 10 min prior to sacrifice. Insulin stimulation significantly increased phosphorylation of IRS-1 at Tyr612 and AKT at both Ser473 and Thr308, and a trend to increase phosphorylation of IR at Tyr1146, IRS-1 at Tyr612, mTOR at Ser2448 and p70S6K at Thr421/424 (Fig 4.5). SRL treatment reduced phosphorylation of all studied insulin signaling proteins, while CsA group did not affect phosphorylation of any of the proteins. Total proteins were not changed in any of the treatments (Fig.4.5).

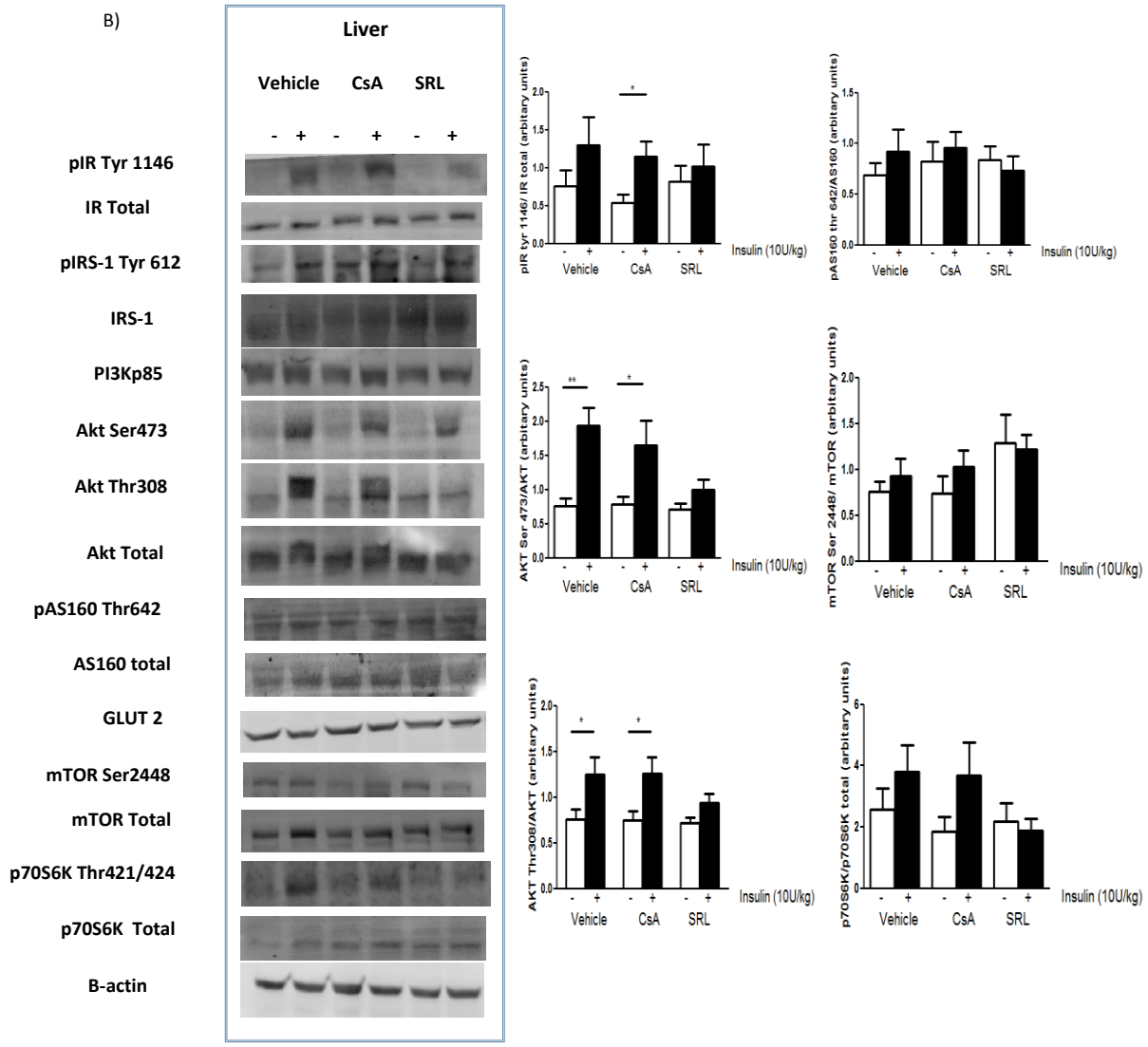


Figure 4.5. Expression of proteins of the insulin signaling pathway in liver after 3 week-treatment period with CsA or SRL. Phosphorylation levels of IR Tyr1146, IRS-1 Tyr612, protein expression levels of PI3K p85 subunit and GLUT2 and phosphorylation levels of pAkt Ser473 and Thr308, p70S6K Thr412/424, mTOR Ser2448, and AS160 Thr642, after stimulation with insulin (B). *p<0.05 vehicle vs. CsA or SRL group; *p<0.05, **p<0.01 basal vs. insulin.

4.2.4 Effect of CsA and SRL on protein and gene expression in muscle

4.2.4.1 SRL decreases PGC1-α in muscle.

SRL treatment reduced PGC1-*α* protein expression in muscle (~50%, p<0.05), compared to vehicle group (Fig. 4.6A), while CsA had no effect. No changes were found on FOXO1 and PTP1B gene and protein expression with both treatments.

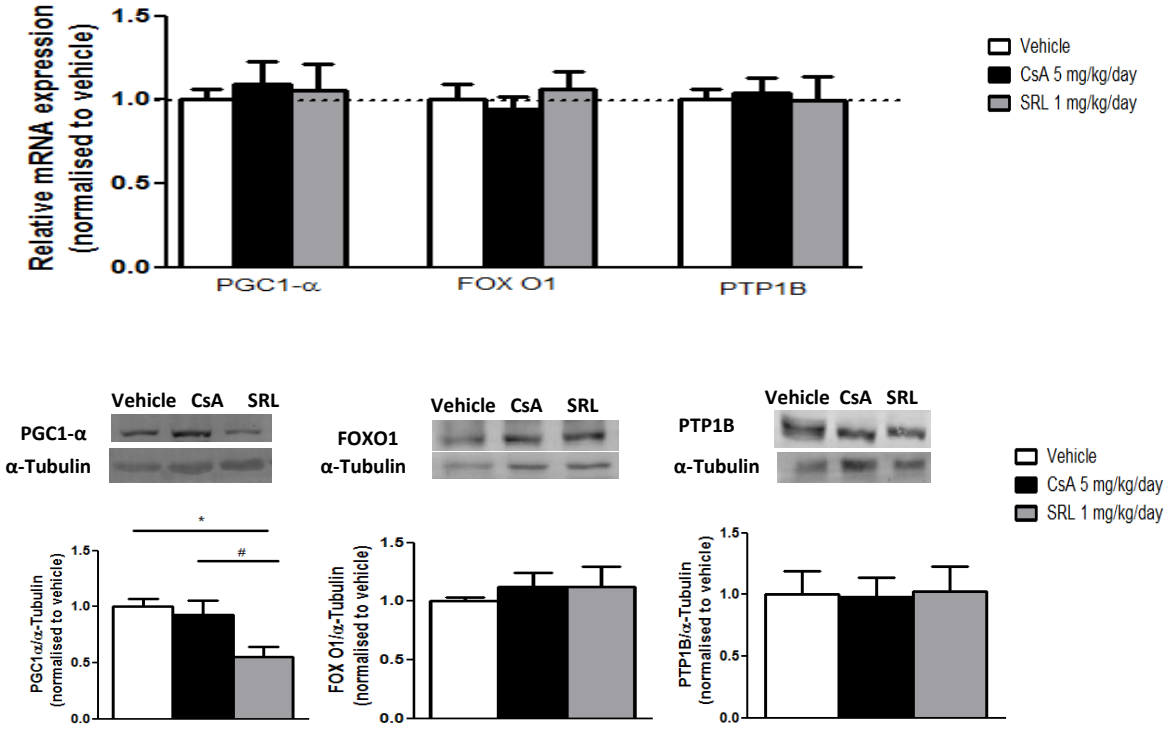


Figure 4.6. PGC1-α, FOXO1, PTP1B gene and protein expression in muscle after 3 weeks-treatment period with CsA or SRL. mRNA relative expression levels were determined by Real-time PCR (n=8) and protein expression levels determined by western blotting (three to five independent experiments) for PGC1-α, FOXO1 and PTPB1. Data are presented as mean ± SEM, *p<0.05 vehicle vs. CsA or SRL group; #p<0.05 CsA vs. SRL group.

4.2.4.2 Effect of CsA and SRL on insulin signaling in muscle

In muscle, no significant changes were found in gene expression of either IRS-1 or GLUT4, while GLUT1 gene expression was increased in the CsA group. However at the protein level, there was a trend for an increase in IRS-1 and a decrease in GLUT1 in the SRL group, while no changes were observed in GLUT4 protein expression in either treated group (Fig. 4.7A).

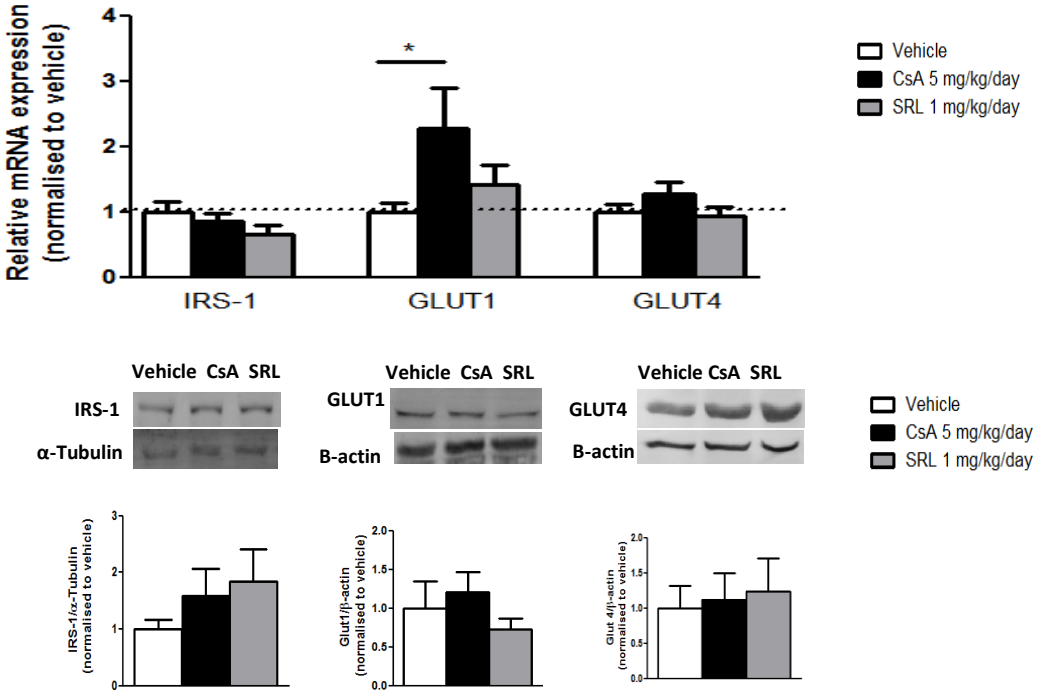


Figure 4.7. Expression of genes and proteins of the insulin signaling in muscle after 3 week-treatment period with CsA or SRL. mRNA relative expression levels were determined by Real-time PCR (n=8) and protein expression levels determined by western blotting (three to five independent experiments) for IRS-1, GLUT1 and GLUT4. Data are presented as mean ± SEM. *p<0.05 vehicle vs. CsA group

To determine whether therapeutic dose of these immunosuppressive agents affects insulin signaling in muscle, the phosphorylation of important key players were assessed. Insulin stimulation significantly increased phosphorylation of IRS-1 at Tyr612 and Akt at both Ser473 and Thr308 (Fig 4.7B). SRL treatment reduced phosphorylation of IRS-1 on Tyr612, AKT at Thr308, mTOR at Ser2448 and p70S6K at Thr421/424, compared with the vehicle group. On the other hand, while no changes were observed for Akt phosphorylation on Ser473, Akt Thr308 phosphorylation was impaired by CsA. Total proteins were not changed in any of the treatments (Fig. 4.7B).

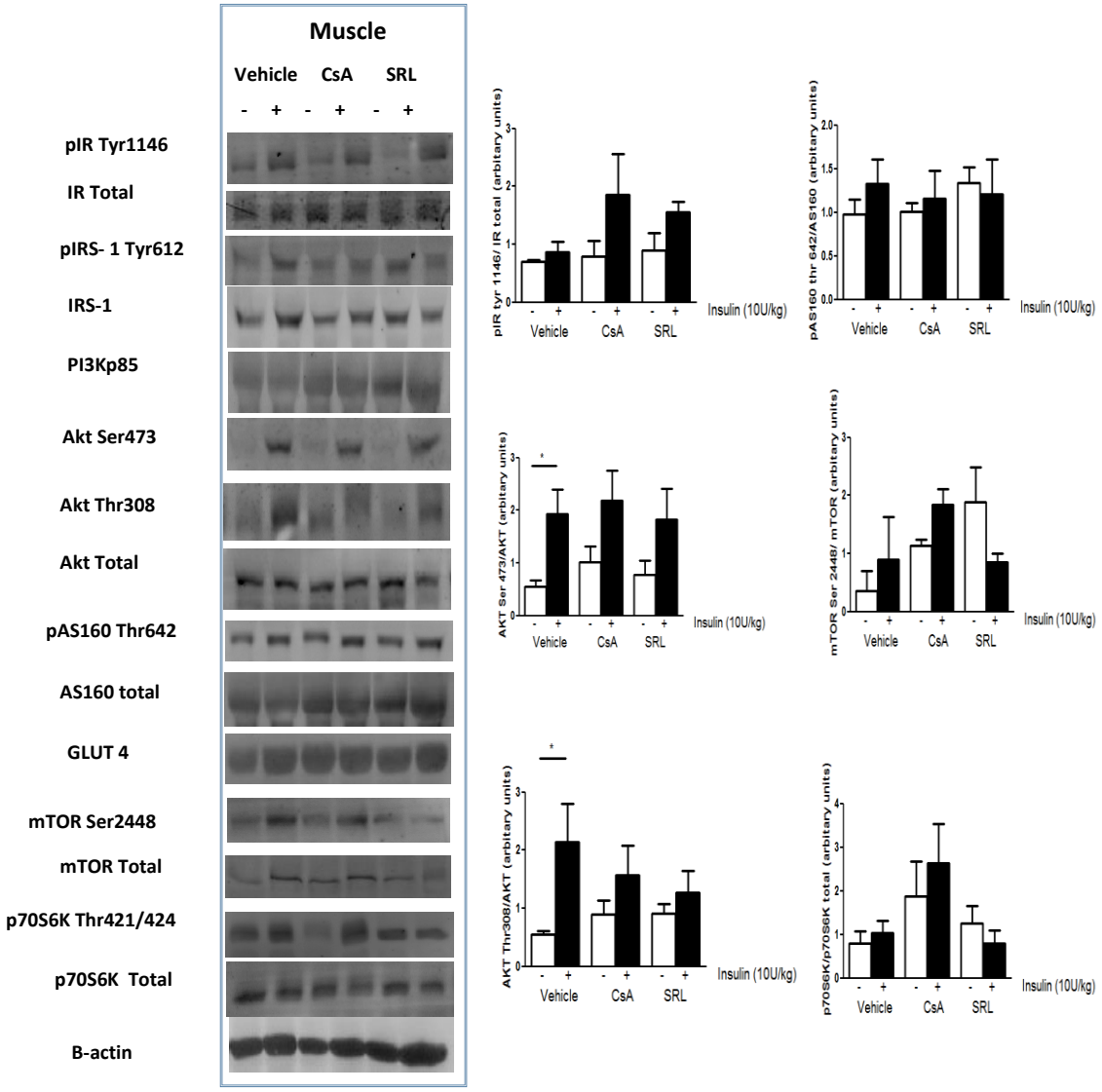


Figure 4.8. Expression of proteins of the insulin signaling pathway in muscle after 3 week-treatment period with CsA or SRL. Phosphorylation levels of IR Tyr1146, IRS-1 Tyr612, protein expression levels of PI3K p85 subunit, GLUT4, and phosphorylation levels of p70S6K Thr421/424, mTOR Ser2448, and AS160 Thr642, after stimulation with insulin, were determined by western blotting (three to five independent experiments) (B). Data are presented as mean ± SEM. *p<0.05 vehicle vs. CsA group; *p<0.05 basal vs. insulin

4.2.5 Effect of CsA and SRL on protein and gene expression in adipose tissue

4.2.5.1 Neither CsA nor SRL affected PTP1B, PGC1-α, or FOXO1 protein levels in perirenal adipose tissue

In perirenal adipose tissue, although SRL treatment reduced gene expression of PGC1-α (61%, p<0.05) compared to vehicle group, the protein levels were similar between

the groups. Moreover, no changes were found on either FOXO1 or PTP1B gene or protein expression levels in this tissue (Fig. 4.9A).

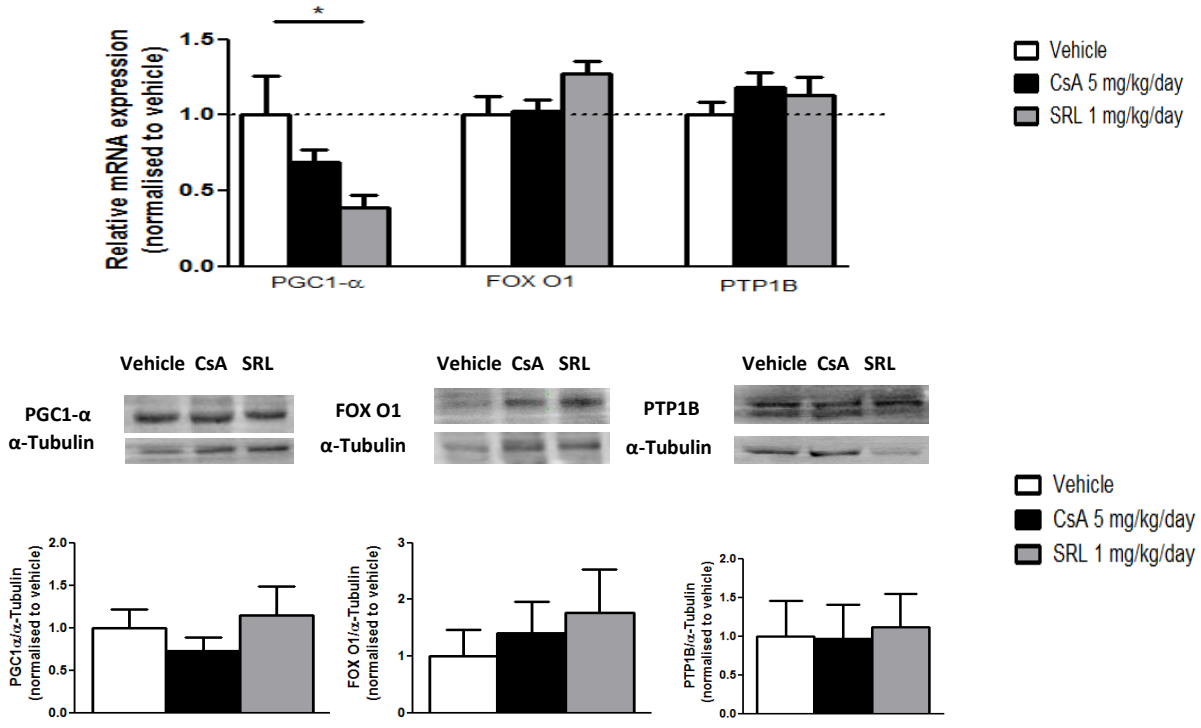


Figure 4.9. PGC1-α, FOXO1, PTP1B gene and protein expression in perirenal adipose tissue after 3 week-treatment with CsA or SRL. mRNA relative expression levels were determined by Real-time PCR (n=8) and protein expression levels determined by western blotting (three to five independent experiments) for PGC1-α, FOXO1 and PTP1B. Data are presented as mean ± SEM , *p<0.05 vehicle vs. SRL group.

4.2.5.2 Effects of CsA and SRL on insulin signaling in adipose tissue

In perirenal adipose tissue, while no changes were observed in IR and IRS-1 gene expression (Fig. 4.10), there was a trend to increase its protein levels in the SRL treated group. On the other hand, GLUT1 and GLUT4 had a trend to decrease gene expression, while its protein levels were not changed in the SRL group. CsA treatment did not affect gene or protein expression of IR, IRS-1, GLUT1 or GLUT4 (Fig. 4.10).

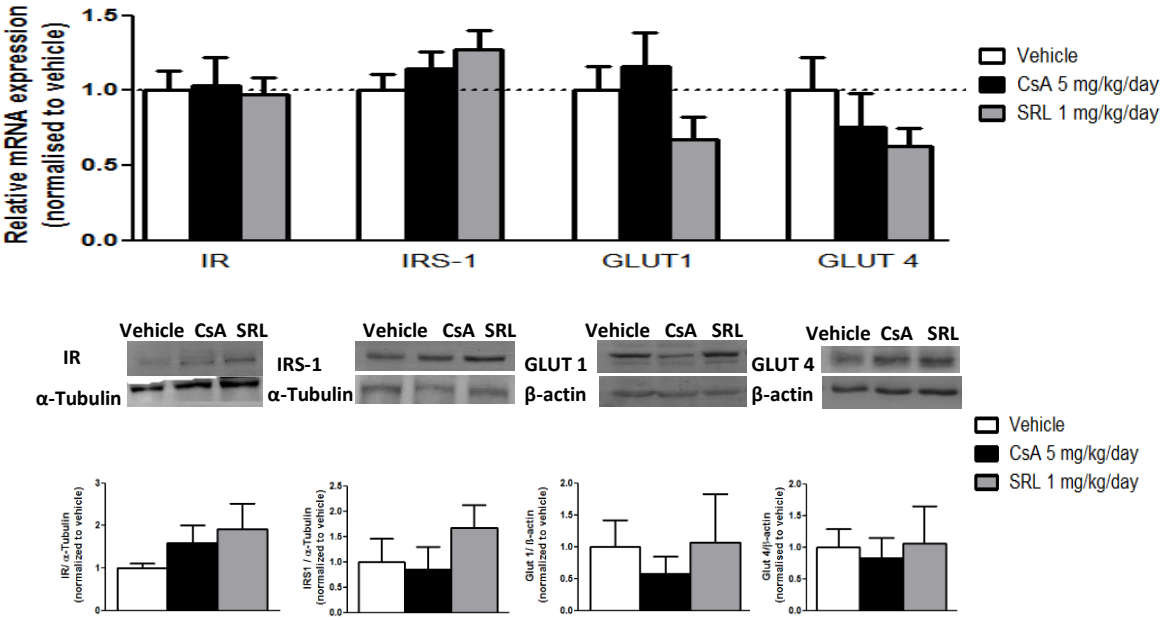


Figure 4.10. Expression of genes and proteins of the insulin signaling in epididymal adipose tissue after 3 week-treatment period with CsA and SRL. mRNA relative expression levels were determined by Real-time PCR (n=8) and protein expression levels determined by western blotting (three to five independent experiments) for IRS-1, GLUT1 and GLUT4. Data are presented as mean ± SEM.

In epididymal adipose tissue, insulin stimulation significantly increased phosphorylation of IR at Tyr1146, IRS-1 at Tyr612 and AKT at Ser473 (Fig 4.11). Treatments with both CsA and SRL significantly impaired phosphorylation of IR Tyr1146 residue compared to vehicle. On the other hand, SRL treatment reduced AKT phosphorylation at Ser473, mTOR at Ser2448 and p70S6K at Thr421/424, compared with the vehicle group (Fig. 4.11). Total proteins were not changed in any of the treatments.

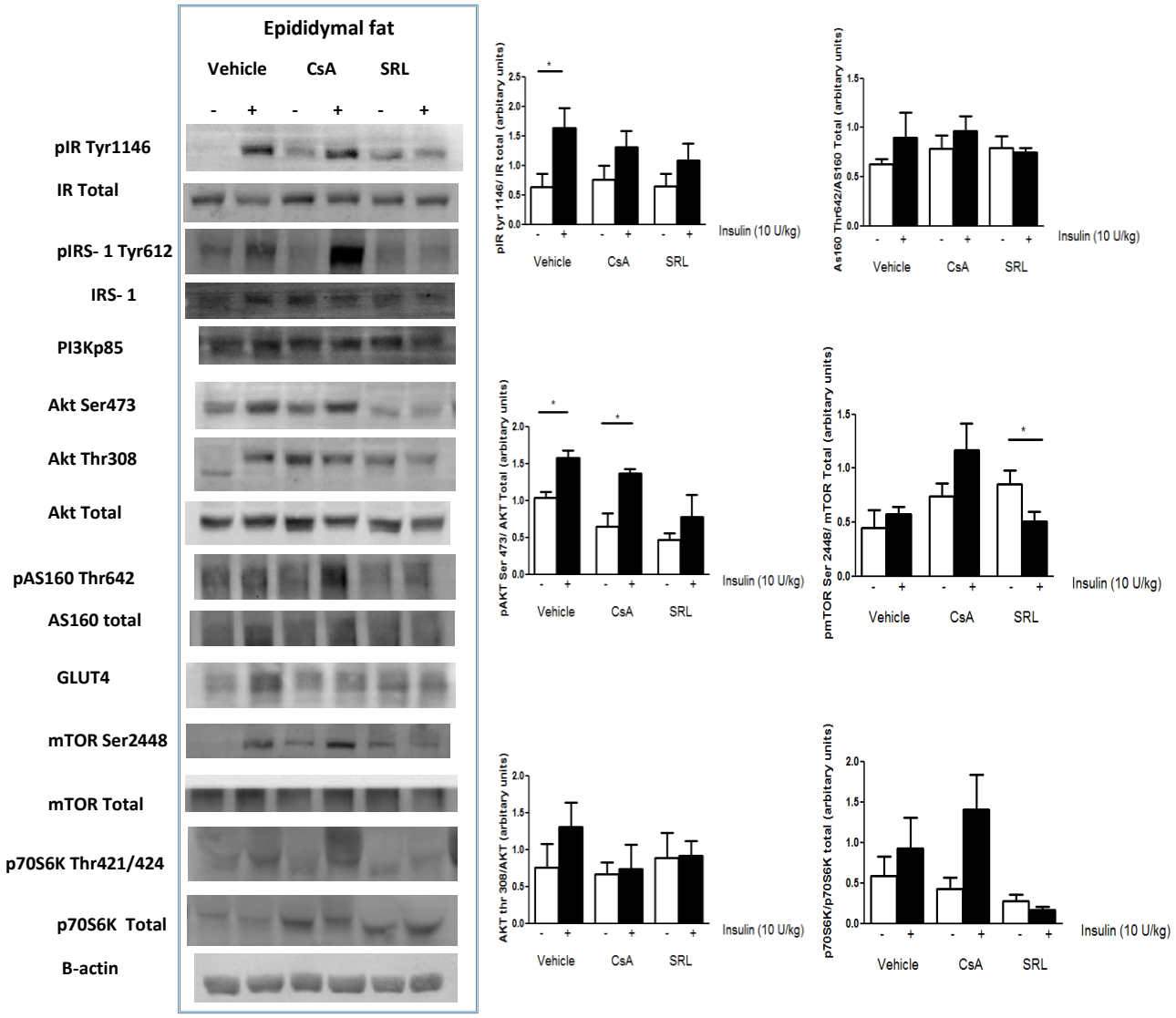


Figure 4.11. Expression of genes and proteins of the insulin signaling pathway in epididymal adipose tissue after 3 week-treatment period with CsA and SRL. Phosphorylation levels of IR Tyr1146, IRS-1 Tyr612, protein expression levels of PI3K p85 subunit, GLUT4 and phosphorylation levels of p70S6K Thr421/424, mTOR Ser2448, and AS160 Thr642, after stimulation with insulin, were determined by western blotting (three to five independent experiments). Data are presented as mean ± SEM, *p<0.05 basal vs. insulin.

4.3 Discussion

The present study indicates that an *in vivo* 3 week-treatment of Wistar rats with either CsA or SRL impairs glucose metabolism. Treatment with CsA resulted in impaired glucose tolerance and insulin sensitivity as demonstrated during a GTT and an ITT, respectively. Moreover, treatment with CsA increased protein expression of key enzymes for hepatic gluconeogenesis, G6Pase and PEPCK, and the upstream transcription factors PGC1-α and FOXO1 were increased in the liver, providing an insight into the molecular mechanisms for

the elevation of glucose on the blood. Moreover, PTP1B protein levels were also increased in the liver in the CsA-treated group, which may contribute to impaired insulin sensitivity observed during the treatment. Although SRL had no effect on the expression of genes or proteins involved in gluconeogenesis in the liver, it significantly decreased GK protein expression, an enzyme responsible for the phosphorylation of glucose into glucose-6-phosphate possibly leading to impaired production of glycogen. In addition, the effect of these agents on activation of insulin signaling in the liver, muscle and adipose tissue were evaluated. SRL treatment reduced Akt phosphorylation in these tissues, then leading to reduced AS160 phosphorylation. These effects combined might impair GLUT4 translocation, which we did not measured for lack of tissue, explaining the reduction in glucose uptake observed previously (Lopes *et al.*, 2013a). Altogether, these results suggest that CsA and SRL modulate glucose metabolism and insulin action, although through different targets, i.e. while CsA seems to enhance gluconeogenesis, SRL mainly impairs insulin signaling in peripheral tissues. These effects might contribute to the development of insulin resistance and NODAT observed during immunosuppressive therapy.

During a GTT, the CsA group presented impaired glucose tolerance when compared to either the vehicle or the SRL-treated groups, during a GTT. The latter, also presented an impaired glucose excursion curve. As normal insulin action is required for clearing an oral glucose load (Ferrannini *et al.*, 1985), this impairment might be due to reduced insulin secretion by β-cells and/or a reduction in peripheral insulin sensitivity (Hjelmesaeth *et al.*, 2007; Øzbay *et al.*, 2011) in the CsA-treated group. This was confirmed after an ITT, as even when an exogenous insulin bolus was administered, the glucose levels in the CsA group remained higher, and the rate of glucose disposal to reach basal levels was slower. This is also true for the SRL group, in particular at the 60 minutes time point where the glucose values were significantly higher than vehicle and closer to the CsA group. In fact, the presence of higher levels of insulin was not sufficient to decrease glucose levels similar to the levels observed in the vehicle group, suggesting marked insulin resistance in both CsA and SRL treated groups. Furthermore, to evaluate if this could be due to impaired insulin secretion from the β-cells of the islets of Langerhans, after 3 weeks of treatment with therapeutic doses, we measured insulin and C-Peptide levels. However, no differences were observed for insulin, and even thought C-peptide levels were reduced, it was not significant. This condition is usually associated with induced diabetes in rats (Amin *et al.*, 2011) and a defect in β-cells (Palmer *et al.*, 2004).

SRL is considered to be less nephrotoxic than CsA, and is presently a valid option to calcineurin inhibitors for the maintenance of immunosuppression (Klawitter *et al.*, 2009). Therefore, we also wanted to evaluate if the clearance of glucose rate in urine was impaired. Surprisingly, we found a tendency for an increase in glucose clearance rate in the SRL treated group, which might be related to an increase of glucose in the urine, in greater quantities than the renal tubule can absorb (glycosuria), and this condition has already observed in patients under SRL therapy (Franz *et al.*, 2010). No difference was observed in the CsA group, but as Yale *et al.* (1985) have shown, it requires higher doses and duration of treatment to cause glycosuria with CsA (10 mg/kg BW/day for 12 weeks).

Moreover, as the development of insulin resistance has been linked to enhanced hepatic gluconeogenesis, we evaluated some of the key markers of this pathway. In our model, after 3 weeks of treatment with CsA, G6Pase protein levels were significantly increased and were accompanied by a tendency for an increase in protein expression for PEPCK and transcription factors PGC1-α and FOXO1, confirming an overstimulated hepatic gluconeogenesis. In the SRL group, we did not observe an increase in gluconeogenesis, as reported previously by Houde *et al.* (2010) and Lamming *et al.* (2012). This apparent discrepancy might be dose-related, as the authors used a higher dose of SRL. Interestingly, although no change were observed in GK gene expression, an enzyme responsible for producing glucose-6-phosphate, its protein level was significantly decreased in the SRL group, suggesting the glycogen production was decreased in the SRL-treated group. Glycogen storage is usually decreased in patients with type 2 diabetes and might be a consequence of insulin resistance (Saltiel, 2001). In addition, GK is controlled at a transcriptional level in a TORC1-dependent manner (Dai *et al.*, 2013) and therefore assays to determine GK activity should be considered in future studies with SRL treatment. Moreover, gene and protein levels for PGC1-α and FOXO1 were also measured in muscle and perirenal adipose tissue, where their actions are more linked to their role in mitochondrial biogenesis, myogenesis and adipocyte differentiation (Amat *et al.*, 2009; Liang *et al.*, 2006). PGC1-α expression is directly related with insulin sensitivity and is down regulated in muscle of type 2 diabetic subjects (Liang *et al.*, 2006). Therefore, a reduction in PGC1-α expression in the muscle of SRL-treated rats may account for the development of insulin resistance. Moreover, muscle specific mTORC1 loss is associated with a decrease in PGC1-α and with a reduction the expression of mitochondrial target genes including PGC-1α itself and in oxidative metabolism (Bentzinger *et al.*, 2008; Cunningham *et al.*, 2007; Laplante *et al.*, 2012; Romanino *et al.*, 2011).

Since PTP1B is a negative regulator of insulin signaling, and its deletion has been coupled with improved insulin sensitivity, we evaluated how the *in vivo* treatment with these agents could affect its gene and protein expression in the various tissues. Interestingly, PTP1B protein level was increased in the CsA group in the liver but not in muscle or adipose tissue. Although PTP1B gene expression in the liver was increased by SRL treatment, no changes were observed in protein expression in liver, muscle or adipose tissue. To our knowledge this is the first report showing alterations on PTP1B protein expression with CsA treatment. This increase in PTP1B protein expression may be linked to an increase in insulin resistance and gluconeogenesis as liver specific PTP1B $^{-/-}$ mice have been shown to have decreased expression of gluconeogenic genes and increased hepatic insulin signaling (Delibegovic *et al.*, 2009), and were able to reverse glucose intolerance (Owen *et al.*, 2013). Assays to determine PTP1B activity should be considered in future studies with CsA treatment, as in diabetic rats, increased PTP1B levels and activity, decrease glucose uptake and insulin signaling (Wu *et al.*, 2005).

To further elucidate the development of whole body glucose intolerance and the previous reported data showing that treatment with CsA or SRL impairs insulin-stimulated glucose uptake in epididymal adipose tissue (Fuhrmann *et al.*, 2014; Lopes *et al.*, 2013a), we also analyzed protein expression and activation of important insulin signaling markers in \muscle, liver and adipose tissue. While IRS-1 protein levels were significantly increased in the liver, and tended to be increased in muscle and adipose tissue, a reduction in GLUT1 protein level was detectable in liver and muscle with the SRL treatment. No changes were observed in GLUT2 (liver) or GLUT4 (muscle and adipose tissue), the main insulin-stimulated transporter (Leto *et al.*, 2012; Pessin *et al.*, 2000), with either treatments. A decrease in GLUT1 protein expression with the SRL treatment explain the reduction of the basal glucose uptake, observed by Pereira *et al.* (2012), Fuhrmann *et al.* (2014) and Deblon *et al.* (2012), while the increase in IRS-1 expression also observed by Takano *et al.* (2001) and Um *et al.* (2006) might be a compensatory mechanism. In this study we cannot exclude the possibility that even though the GLUT4 protein expression was not different, its translocation to the membrane could be impaired. This experiment was not performed due to the lack of tissue, but should be addressed in future studies. Nonetheless, impaired glucose uptake in CsA-treated rats might be related with a reduced amount of GLUT4 in the plasma membrane as Pereira *et al*. (unpublished data) recently demonstrated that CsA treatment reduced the insulin-stimulated presence of GLUT4 in the plasma membrane of differentiated

human pre-adipocytes and L6 muscle cells. On the other hand, in the SRL group glucose uptake might be decreased due to an impairment of the insulin signaling, as already demonstrated in human and rat insulin sensitive cells (Kumar *et al.*, 2010; Pereira *et al.*, 2012; Sarbassov *et al.*, 2006; Shivaswamy *et al.*, 2013). Moreover, the decrease in PGC1α-protein expression in the muscle of the SRL-treated group might also be responsible for a decrease in insulin sensitivity, as PGC1-α increases the expression of the insulin-sensitive transporter GLUT4 in the muscle (Baar *et al.*, 2002; Michael *et al.*, 2001). Insulin stimulation initiates intracellular signaling when it binds to the insulin receptor, phosphorylating its tyrosine residues. In our work, phosphorylation of the insulin receptor at Tyr1146 residue was decreased in the SRL group both in liver and adipose tissue. Moreover SRL also impaired phosphorylation of the key factor, Akt at Ser473 and Thr308 residues in liver and adipose tissue, while no alterations were observed by the CsA treatment, previously demonstrated both *in vitro and in vivo* (Bodine *et al.*, 2001; Di Paolo *et al.*, 2006; Lungu *et al.*, 2004). Sarbassov *et al.* (2005) have also shown that mTOR kinase and rictor are essential for phosphorylation of Akt Ser473 and SRL reduces insulin phosphorylation of IRS-1 on Tyr residues (Danielsson *et al.*, 2005), which is in accordance with our results at least in muscle. Moreover, Shivaswamy *et al.* (2013) observed recently that SRL treatment reduces insulin-stimulated phosphorylation of Akt in liver, muscle and fat. On the other hand, insulin sensitivity may also be affected by intracellular lipid accumulation, through impairment of IRS-1-PI3K-Akt signaling pathways (Di Paolo *et al.*, 2006; Morino *et al.*, 2005), which may also be the case, as our group already demonstrated that after 3 weeks of treatment with SRL, there is an accumulation of TGs in liver and muscle (Lopes *et al.*, in press). Impaired Akt activation leads also to a decrease in phosphorylation of AS160, an important substrates of Akt that controls the translocation of glucose transporters to the plasma membrane. These data, in accordance with other studies (Deblon *et al*., 2012; Pereira *et al*., 2012; Pereira *et al*.; unpublished data) reveal that SRL treatment inhibits activation of Akt in response to insulin and affect glucose metabolism in skeletal muscles and adipocytes. As expected, the mTOR pathway was blocked by SRL treatment, as evidenced by the lack of phosphorylation of its downstream target, the p70S6K.

Taken together, these data indicate that CsA affects glucose metabolism, by increasing gluconeogenesis in liver and SRL mainly by impairing the insulin signaling cascade pathway in peripheral tissues, which ultimately can affect glucose uptake (Fig.4.12). These effects might contribute to the development of insulin resistance after

immunosuppressive therapy, and caution is required when choosing the therapy to apply to patients, in order to prevent the development of NODAT.

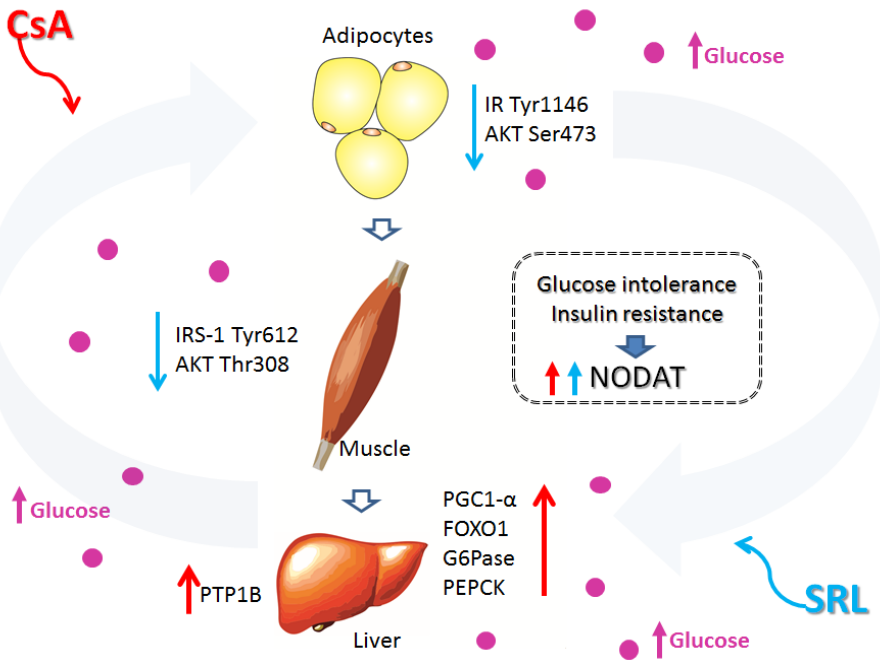


Figure 4.12. Scheme summarizing the effects of CsA and SRL on the gluconeogenesis and insulin signaling in muscle and adipose tissue. Red arrows correspond to CsA; Blue arrows correspond to SRL. ↑, increase; ↓, decrease.

Chapter 5

Short and long term effects of cyclosporine A and sirolimus *in vivo* on genes and proteins involved in lipid metabolism in Wistar rats

This Chapter comprises the work accepted in *Metabolism, Clinical and Experimental* (2014) by **Lopes PC**, Fuhrmann A, Sereno J, Espinoza DO, Pereira, MJ, Eriksson JW, Reis F, Carvalho E

5.1 Introduction

Cyclosporine A and sirolimus are immunosuppressive agents used to prevent allograft rejection after transplantation and as therapies for autoimmune diseases. Both agents are associated with serious long-term complications, including dyslipidemia and new onset of diabetes after transplantation (NODAT) (Gueguen *et al.*, 2007; Gueguen Y, 2004; Miller, 2002). Dyslipidemia leads to the development of atherogenesis and post-transplant coronary artery disease, the most common cause of morbidity and mortality among transplant patients (Bumgardner *et al.*, 1995; Markell *et al.*, 1989). Dyslipidemia generally appears in the first year post-transplant and persists regardless of dietary modifications, occurring in up to 60% of renal (Badiou S, 2009) and 45% of liver transplant patients (Rossetto *et al.*, 2010). Immunosuppressive therapy is associated with increased serum levels of triglycerides (TG), total cholesterol, low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), free-fatty acids (FFA) and apolipoprotein B, in a dose-dependent manner (Ichimaru *et al.*, 2001; Morrisett *et al.*, 2002; Spinelli *et al.*, 2011). Nonetheless, the mechanisms that promote these effects of CsA and SRL are not completely known.

Lipids are stored in adipose tissue, for future utilization, under the control of regulatory factors, such as insulin, catecholamines and autonomic nervous system mediators (Large *et al.*, 2004). Energy stored in adipocytes is regulated by a balance between TG storage, via esterification of FFA and lipogenesis, and release of FFA and glycerol, via

lipolysis. In the fed state, increase in blood insulin, glucose and lipid levels promotes lipogenesis (Large *et al.*, 2004), while in the fasted state, intracellular lipases are responsible for lipolysis (Viscarra *et al.*, 2013). In the presence of low glucose, fatty acids become a major energy source for peripheral tissues. In contrast, during glucose availability, lipogenic genes are activated, including Acetyl-CoA carboxylases 1 and 2 (ACC1 and ACC2), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD1) and, consequently, the synthesis of TG through acyl CoA:diacylglycerol acyltransferase (DGAT) enzymes. Glucose and insulin mediate these processes, whereas ACCs are responsible for catalyzing the first step, the synthesis of malonyl-CoA, a substrate for the *novo* fatty acid synthesis and the regulator of fatty acid oxidation. Key transcription factors, like sterol response-elements binding proteins 1 and 2 (SREBP1 and SREBP2), liver X receptors (LXR) and carbohydrate-responsive element-binding protein (ChREBP) are also essential for the maintenance of this cascade (Brown *et al.*, 1997; Davies *et al.*, 2008; Dentin *et al.*, 2004; Iizuka *et al.*, 2004; Wakil *et al.*, 2009; Yen *et al.*, 2008).

Determining the influence of immunosuppressive therapy in the balance between lipolysis and lipogenesis, is important, since elevated levels of fatty acids in circulation may contribute to ectopic fat accumulation in peripheral tissues (Roden *et al.*, 1996). Consequently, the functionality of these tissues can be seriously affected, leading to the pathogenesis of obesity-related conditions, such as insulin resistance, diabetes and cardiovascular diseases. Moreover, we have recently demonstrated that CsA and SRL therapies are associated with insulin resistance and dyslipidemia (Lopes *et al.*, 2013b). The main aim of the present study was to investigate and compare the *in vivo* effects of chronic administration of CsA and SRL on genes and proteins involved in lipid metabolism in adipose tissue and liver, using Wistar rats as a model system.

5.2 Results

5.2.1 Effects of CsA and SRL on lipolysis

CsA and SRL treatment *in vivo* significantly enhanced isolated adipocyte lipolysis during isoproterenol stimulation (1μM) by 5 ($p<0.01$) and 9-fold ($p<0.001$), respectively, compared to the vehicle non-stimulated basal, at 3 weeks (Fig. 5.1). In addition, after 9 weeks, CsA and SRL increased isoproterenol-stimulated lipolysis by 4-fold ($p<0.01$) and 6-fold ($p<0.01$) compared to vehicle non-stimulated, respectively (Fig. 5.1). However, when

the adipocytes were incubated with insulin (1000 µU/mL), CsA or SRL treatment show a tendency to revert the insulin´s antilipolytic effects, but this was not significant. Insulin caused a non-significant reduction of 34% and 18% at 3 and 9 weeks with CsA treatment, and 42% and 30% of reduction at 3 and 9 weeks, respectively, for SRL- treated animals (Fig. 5.1).

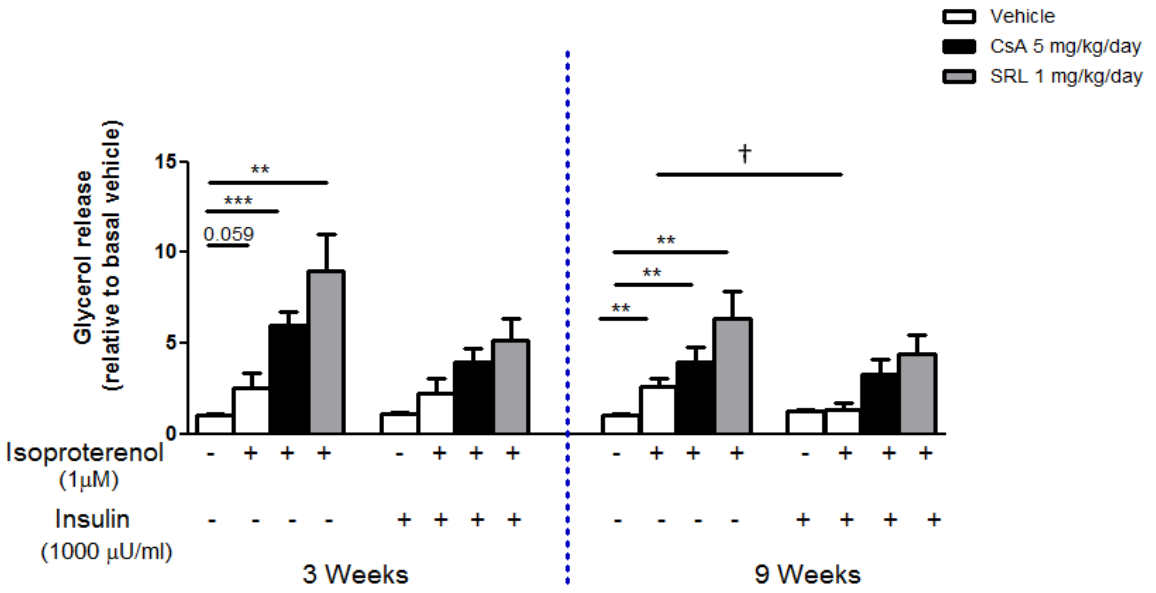


Figure 5.1 - Effects of *in vivo* treatment of Wistar rats with CsA and SRL on lipolysis, in isolated adipocytes. To test the effect of these drugs on lipolysis and the antilipolytic effect of insulin, adipocyte suspension (1:10), was supplemented with isoproterenol (1 µM) and insulin (1000 µU/ml). Glycerol released into the medium was measured by colorimetric absorbance in a Glycerol release kit (Zen – Bio) and used as an index of lipolysis. Lipolysis rate for each condition was calculated relative to basal vehicle for each time point and values are presented as mean ± SEM (n=6/group). **p<0.01, **p<0.001 vehicle vs. treated groups, † p<0.01 basal vs. insulin.

5.2.2 Effects of CsA and SRL on body weight and adipocyte weight and diameter

There were no changes in body weight gained for either CsA or SRL-treated groups compared to vehicle-treated group after 3 weeks. However, after 9 weeks, SRL-treated animals showed significantly lower body weight gain when compared to either vehicle or CsA-treated animals ($p<0.05$) (Fig. 5.2A). Between week 3 and week 9 of treatment, the body weight of vehicle and CsA treated animals increased significantly from 351.2 ± 3.6 to 412.0 ± 10.1 g ($p<0.001$) and from 359.5 ± 5.5 to 420.1 ± 6.9 g ($p<0.001$), respectively, while the body weight of SRL-treated animals increased only from 351.2 ± 5.2 to 380.1 ± 8.1 g (non-statistically significant). Isolated adipocytes weight and diameter were

significantly higher after 9 weeks of CsA treatment (0.59 ± 0.10 µg and 97.7 ± 3.4 µm, respectively) compared to either vehicle (0.35 ± 0.04 µg, $p<0.05$; and 84.7 ±3.4 µm $p<0.01$, respectively) or SRL-treated animals (0.33 ± 0.02 µg, $p<0.01$; and 84.8 ± 2.2 µm, $p<0.01$, respectively) (Fig. 5.2B and C). Representative images of perirenal adipose tissue from vehicle, CsA and SRL treated rats are shown (magnification 40X) (Fig.5.2D).

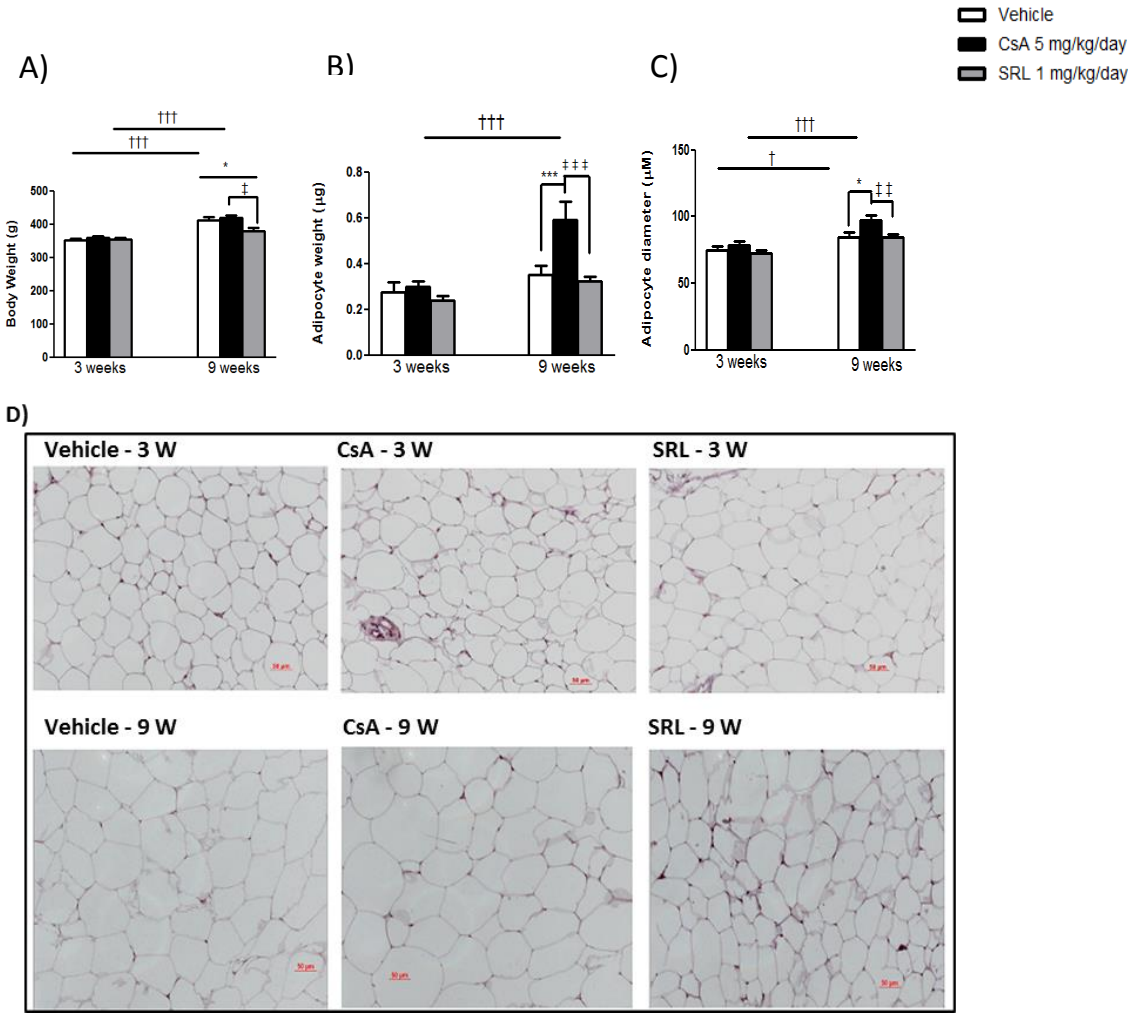


Figure 5.2. Evaluation of total body weight (A), adipocyte weight (B) and diameter (C) after 3 and 9 weeks of treatment with vehicle, CsA and SRL. Representative images of perirenal adipose tissue from vehicle, CsA and SRL treated rats after 3 and 9 weeks (D) (magnification 40X). Data are presented as mean ± SEM (n=8-12/group), *$p<0.05$, ***$p<0.001$ Vehicle vs. treated group, ‡$p<0.05$, ‡‡$p<0.01$,‡‡‡ $p<0.01$ CsA vs. SRL, †$p<0.05$, †††$p<0.001$ between treatments at 3 and 9 weeks.

5.2.3 Effects of CsA and SRL on NEFA and triglycerides

Fed serum NEFA values were increased in the CsA group when compared with the vehicle (1.07 ± 0.25 vs. 0.47 ± 0.04 mmol/l; $p<0.001$) and the SRL-treated group (0.57 ± 0.10 mmol/l; $p<0.01$) (Fig. 5.3A), at 9 weeks. Fed serum TGs, were also increase at 9 weeks for CsA (2.09 ± 0.17

mmol/l) compared to either vehicle or SRL (1.40 ± 0.09 and 1.38 ± 0.10 mmol/l, p<0.001) treatments (Fig. 5.3B). No differences were observed after 3 week for either treatment.

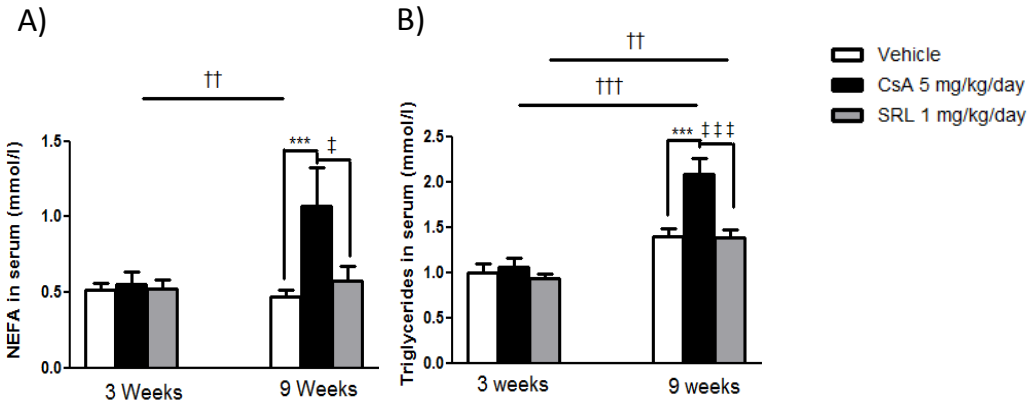


Figure 5.3 – Determination of non-esterified fatty acid (NEFA) (A) and triglycerides (TGs) in serum (B) after 3 and 9 weeks of treatment with CsA and SRL. Data are presented as mean ± SEM (n=8-12/group), ***p<0.001 Vehicle vs. treated group, ‡p<0.05, ‡‡‡ p<0.01 CsA vs. SRL, ††p<0.01, †††p<0.001 between treatments at 3 and 9 weeks.

5.2.4 Effects of CsA and SRL on triglycerides in liver and muscle

Moreover, ectopic deposition of TGs was significantly increased in the SRL-treated rats compared with the vehicle-treated animals in liver (5.3 ± 0.3 µg/mg of tissue, p<0.05 vs. 3.3 ± 0.4 µg/mg of tissue) and in muscle (7.3 ± 1.8 µg/mg of tissue, p<0.05 vs. 3.4 ± 0.5 µg/mg of tissue) after 3 weeks (5.4 A and B). Interestingly, accumulation of ectopic TGs decreased after 9 weeks of SRL treatment compared to the same treatment after 3 weeks in liver (5.3 ± 0.3 vs. 4.2 ± 0.7 µg/mg of tissue) and in muscle (3.9 ± 0.9 µg/mg of tissue). In liver, CsA (4.8 ± 0.6 vs. 4.8 ± 0.3 µg/mg of tissue) and the vehicle groups (3.3 ± 0.4 vs. 3.7 ± 0.9 µg/mg of tissue) maintained their TG levels after 3 and 9 weeks of treatment (Fig. 5.4C). On the other hand, TG levels in muscle were decreased after 9 weeks for CsA (5.9 ± 0.9 vs. 3.3 ± 0.9 µg/mg of tissue) and maintained in vehicle group (3.5 ± 0.5 vs. 4.1 ± 0.6 µg/mg of tissue) (Fig. 5.4 A and B).

Representative images of oil Red O liver stains are shown as demonstrating levels of the triglycerides in the liver and muscle for vehicle, CsA and SRL-treated rats (Fig. 5.4 C and D).

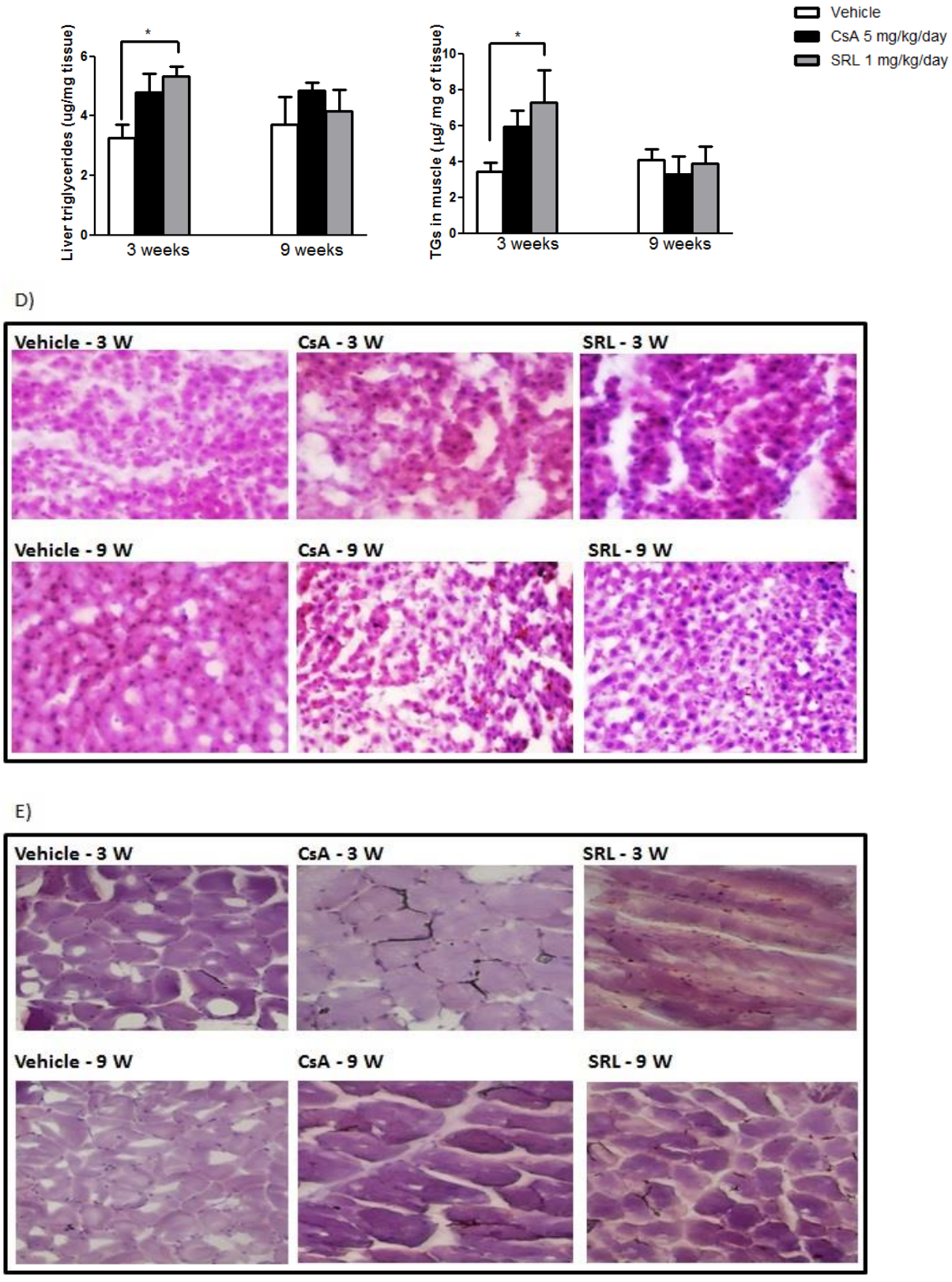


Figure 5.4 – Determination of triglycerides in liver (A) and muscle (B) tissue after 3 and 9 weeks of treatment with CsA and SRL. Rat liver and muscle sections were stained with Oil Red O for confirmation of lipid deposition (C and D). TGs in tissue were obtained by the Folch method and measured using a triglyceride colorimetric assay kit. Data are presented as mean ± SEM (n=8-12/group), *$p<0.05$ vehicle vs. treated group.

5.2.5. Gene expression of markers involved in regulating lipolysis

In order to better understand how immunosuppressive therapy causes hyperlipidemia, we studied the expression of lipolytic genes in perirenal adipose tissue of Wistar rats, after 3 and 9 weeks of treatment with either CsA or SRL. Hormone sensitive lipase (HSL) gene expression was significantly decreased by SRL by about 35% compared with CsA ($p<0.05$). There were no significant differences in HSL after 9 weeks of treatment for either group (Fig. 5.5B). No significant differences were observed for either adipose triglyceride lipase (ATGL) or perilipin A, between the groups (Fig. 5.5 A, C).

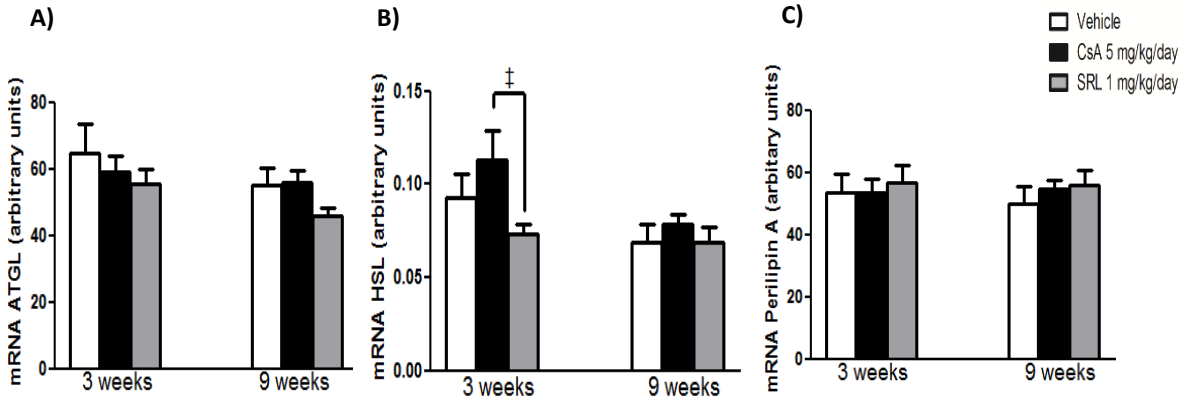
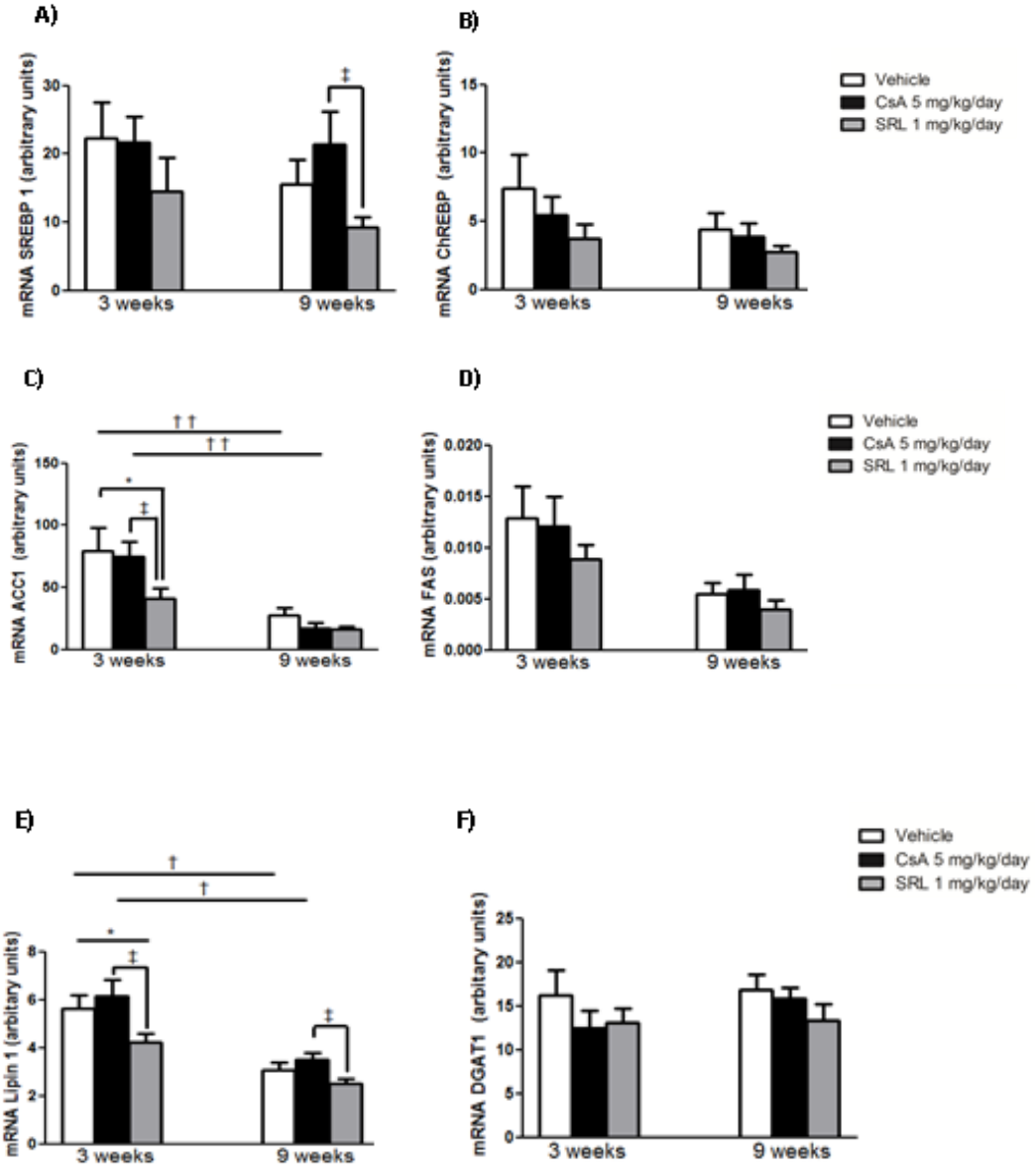


Figure 5.5. Expression of lipolytic genes in perirenal adipose tissue by CsA and SRL. mRNA was extracted from perirenal adipose tissue to measure mRNA expression for ATGL (A), HSL (B), Perilipin A (C). Data are presented as mean ± SEM (n=8-12/group), ‡ $p<0.05$ CsA vs. SRL.

5.2.6 Gene expression of lipogenic factors in adipose tissue

In addition, we studied the expression of key lipogenic genes in perirenal adipose tissue. SRL decreased ACC1 gene expression by 50% compared to vehicle and CsA treatment ($p<0.05$) after 3 weeks (Fig. 5.6C). In addition, after 9 weeks of treatment, ACC1 expression was decrease by more than 50% in all groups, compared to 3 weeks (Fig. 5.6C). Furthermore, SRL also down-regulated Lipin 1 (25%, $p<0.05$), PPAR-γ (42%, $p<0.05$), and decreased SCD1 gene expression (80%, $p<0.001$) compared to vehicle at 3 weeks (Fig. 5.6E, I and J). Differences were lost after 9 weeks of treatment. On the other hand, SREBP, ChREBP, FAS, DGAT1,CD36 and the LPL genes did not change with either treatment

compared to vehicle (Fig. 5.6A, B, D, F, G and H). No alterations were observed with CsA at either 3 or 9 weeks.



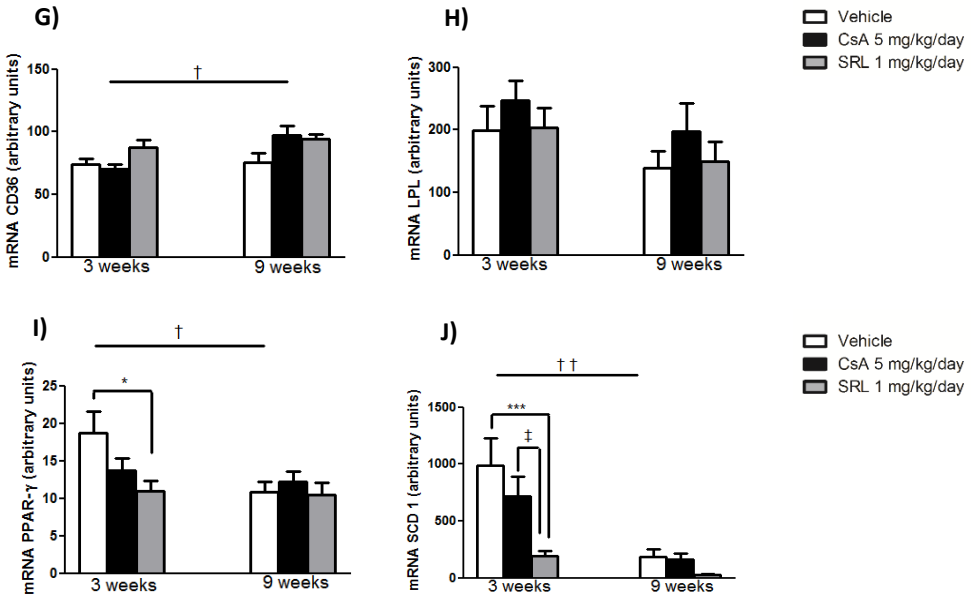


Figure 5.6. Expression of lipogenic genes in perirenal adipose tissue by CsA and SRL. mRNA was extracted from perirenal adipose tissue to measure mRNA expression for SREBP (A), ChREBP (B), ACC1 (C), FAS (D), Lipin 1 (E), DGAT1 (F), CD36 (G), LPL (H), PPAR-γ (I) and SCD1 (J). Data are presented as mean ± SEM (n=8-12/group), *p<0.05, ***p<0.001 Vehicle vs. treated group, ‡p<0.05 CsA vs. SRL, †p<0.05, ††p<0.01 between treatments at 3 and 9 weeks.

5.2.7 Protein expression of factors involved in lipolysis and lipogenesis in adipose tissue

ACC1 protein levels were greatly increased in perirenal adipose tissue with CsA treatment at 3 weeks compared to vehicle (400%, p<0.01) and to SRL (430%, p<0.01). ACC1 was equally decreased for all groups after 9 weeks, in agreement with the gene expression results (Fig. 5.7C). In addition, FAS protein expression was increased although not significantly for CsA treatment compared to vehicle and to SRL (135%, p<0.05) at 3weeks, while there were no significant changes after 9 weeks (Fig. 5.7D). Moreover, HSL expression was significantly increased after 9 weeks with CsA compared to vehicle (115%, p<0.05) and SRL (134%, p<0.01) treatment, although no changes where observed at 3 weeks (Fig. 5.7F). However, neither CsA nor SRL significantly changed SREBP, ChREBP or DGAT1 protein expression (Fig. 5.7A, B and E).

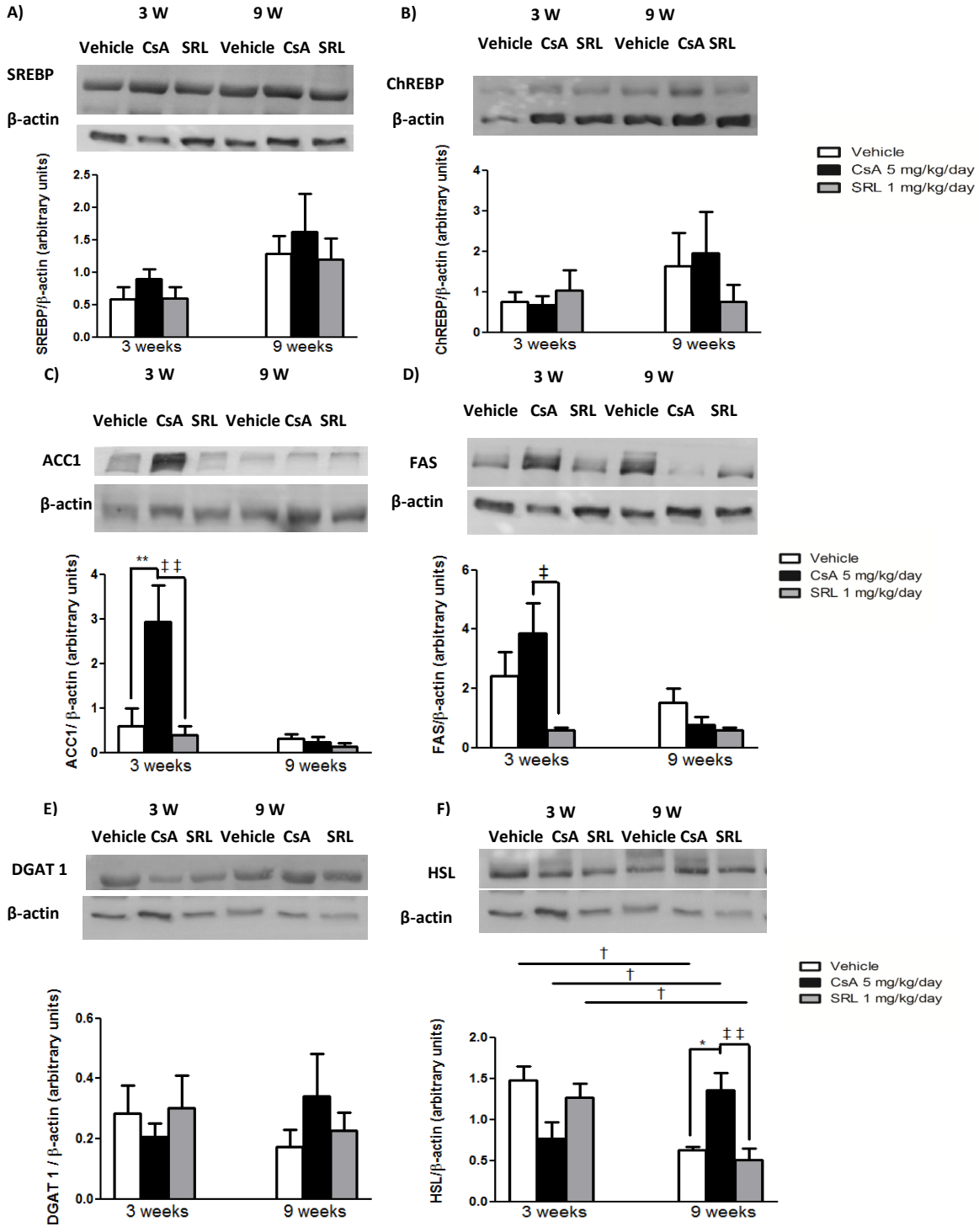


Figure 5.7. Regulation of protein levels of ACC1 (A), FAS (B), SREBP1 (C) and ChREBP (D) DGAT1 (E) and HSL (F) by CsA and SRL. Perirenal adipose tissue was collected as indicated in materials and methods. Total lysates were analyzed by western blotting and results are presented as mean ± SEM of three to five independent experiments. *p<0.05, **p<0.01 Vehicle vs. treated group, ‡p<0.05, ‡‡ p<0.01 CsA vs. SRL, †p<0.05 between treatments at 3 and 9 weeks.

5.2.8 Gene expression of IL-6, TNF-α and adiponectin in perirenal adipose tissue

No significant changes were observed in the IL-6, TNF-α and adiponectin gene expression in perirenal adipose tissue (Fig. 5.8A, B and C).

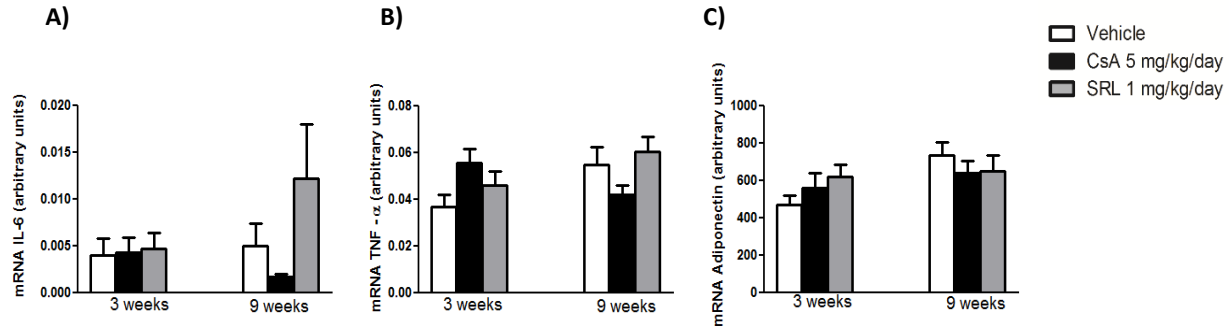
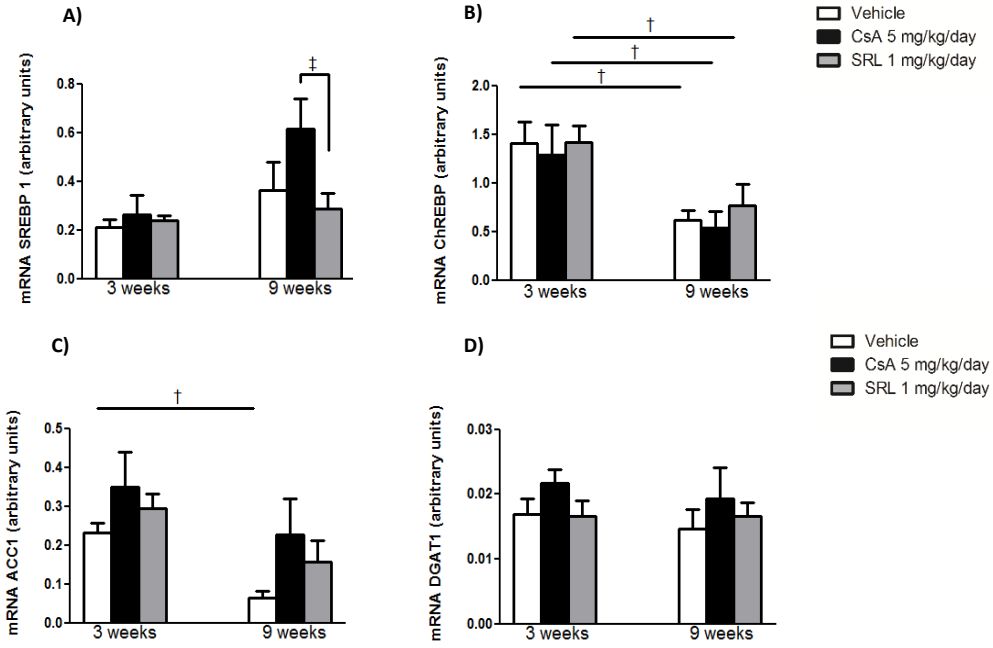


Figure 5.8. Regulation of IL-6 and TNF-α gene expression in perirenal adipose tissue by CsA and SRL. mRNA was extracted from perirenal adipose tissue to measure mRNA expression for IL-6 (A) and TNF-α (B). Data for gene expression are presented as mean ± SEM (n=8-12/group).

5.2.9 Expression of lipogenic factors involved in liver

The expression of the key lipogenic factors ACC1, SREBP1, ChREBP and DGAT1 was also assessed in the liver, after 3 or 9 weeks of treatment with either CsA or SRL. We found no differences for ACC1, SREBP, ChREBP and DGAT1 gene and protein expression at either time point compared to vehicle (Fig. 5.9A, B, C and D) for either treatment, although there was a trend for an increase in ACC1 and SREBP gene expression with CsA treatment at 9 weeks (Fig. 5.9A-H).



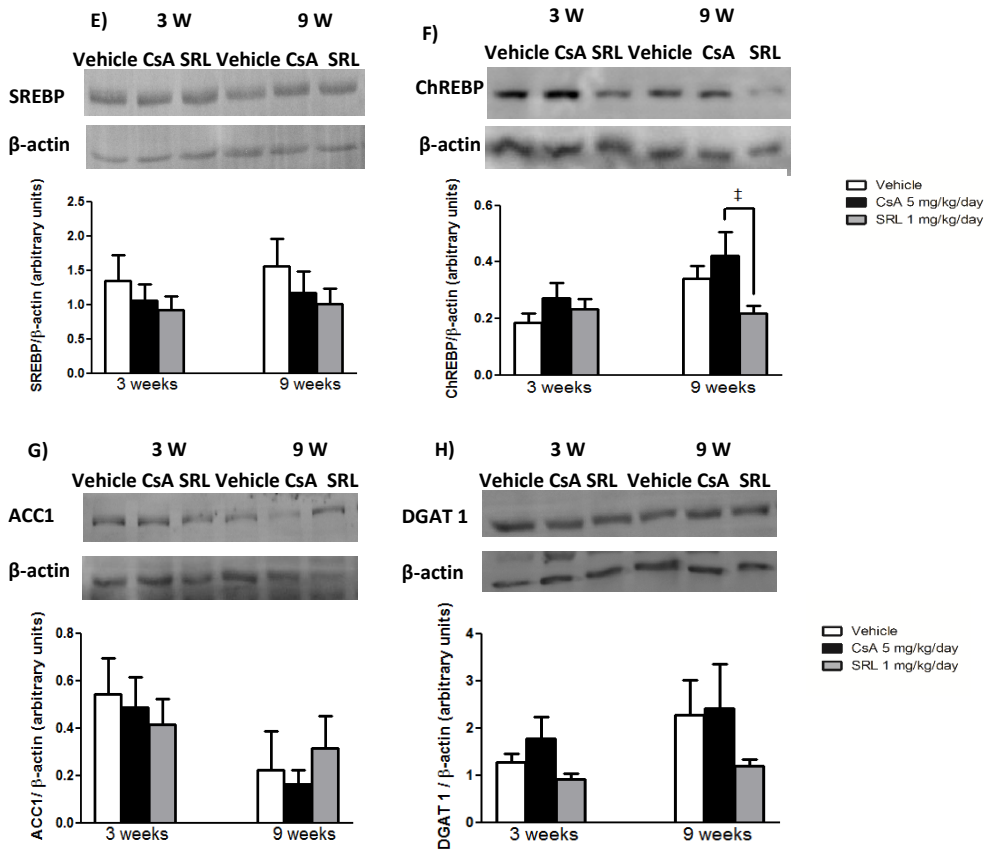


Figure 5.9. Regulation of the expression of lipogenic factors in liver tissue by CsA and SRL. mRNA was extracted from liver tissue to measure mRNA expression for, SREBP1 (A), ChREBP (B), ACC1 (C) and DGAT1 (D). Total lysates of liver were analyzed by western blotting for SREBP1 (E), ChREBP (F), ACC1 (G) and DGAT1 (H) and results are presented as mean ± SEM of three to five independent experiments. Data are presented as mean ± SEM, (=8-12/group), $^{‡}p<0.05$, CsA vs. SRL, $^{†}p<0.05$ between treatments at 3 and 9 weeks.

5.2.10 Gene expression for IL-6 and TNF-α in liver

IL-6 gene expression in the liver was not increased after either 3 or 9 weeks of treatment, compared to vehicle (Fig. 5.10A). On the other hand, TNF-α gene expression in liver was significantly increased by (149%, p<.0.05) in the CsA group at 3 weeks compared to vehicle, but no differences were observed at 9 weeks (Fig. 5.10B).

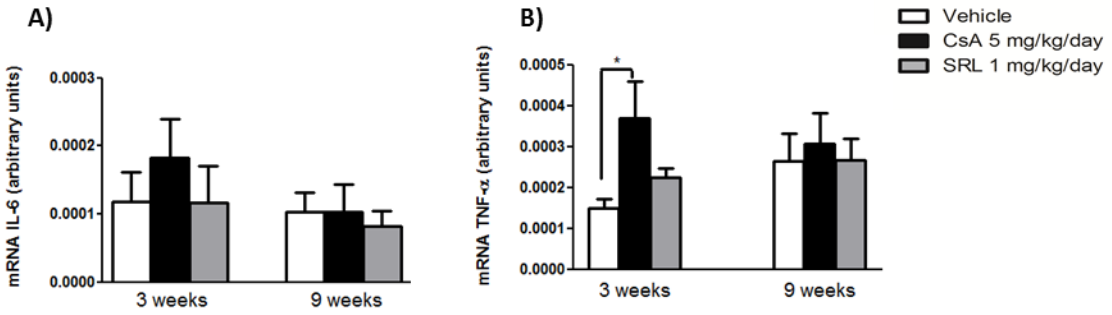


Figure 5.10. Regulation of IL-6 and TNF-α gene expression in liver tissue by CsA and SRL. mRNA was extracted from liver tissue to measure mRNA expression for IL-6 (A) and TNF-α (B). Data for gene expression are presented as mean ± SEM (n=8-12/group), *p<0.05, vehicle vs. CsA.

5.3 Discussion

The present study indicates that *in vivo* treatment of Wistar rats with either CsA or SRL for short or longer periods (3 and 9 weeks), impairs lipid metabolism as shown by gene and protein expression data, as well as by the biochemical and morphological parameters. Treatment with either agents resulted in increased isoproterenol-stimulated lipolysis in adipocytes. CsA increased adipocyte weight and diameter, as well as levels of NEFA and TGs in circulation after 9 weeks, while liver TGs increased with SRL after 3 weeks of treatment. CsA and SRL act on lipogenic and lipolytic pathways differently, affecting them at different time points and acting on different specific target. In this study, CsA effects are more evident after 9 weeks of treatment on lipolytic factors, directly responsible for the increase of lipids in circulation. On the contrary, SRL is more involved in the down-regulation of lipogenic factors (ACC1, lipin1, SDC1 and PPAR-γ) at an early time point, interfering with triglycerides storage in adipocytes. The effects of both IAs suggest that these agents influence the pathway of lipids synthesis through different mechanisms, and thus might contribute to the dyslipidemia observed in rodents and humans after immunosuppressive therapy.

Our results demonstrate that *in vivo* CsA or SRL treatment increases lipolysis in isolated adipocytes. These results are in agreement with a recent study, where *ex-vivo* CsA and SRL treatment of isolated human adipocytes increased isoproterenol-stimulated lipolysis (Pereira *et al.*, 2013) and another where epididymal fat depots from calcineurin, a target of IAs, knockout mice ($CnA\beta^{-/-}$) exhibited a higher phosphorylation of PKA substrates and consequently increased lipolysis under stimulation of isoproterenol (Suk *et al.*, 2013) .

The effects promoted by these immunosuppressive agents on lipolysis may be relevant as impaired FFA mobilization may contribute to dyslipidemia and insulin resistance. Moreover, treatment of rats with either CsA or SRL, at the same doses used here, increased HOMA-IR values, hyperinsulinemia and impaired insulin-stimulated glucose uptake (Lopes *et al.*, 2013b). Lipolysis was only modestly affected by insulin, and even thought CsA or SRL treatment show a tendency to revert the insulin´s antilipolytic effects, this was not significant. A previous report has shown that SRL treatment impairs the insulin antilipolytic effect when insulin is used at lower concentrations (10-100 µU/mL) in human adipocytes (Pereira *et al.*, 2013). It should be noticed that we used a supra-physiological insulin concentration (1mU/mL), and therefore CsA and SRL effects on the antilipolytic effects of insulin at physiological concentrations should be further accessed.

In our rat model, CsA stimulated lipolysis without changing lipolytic and lipogenic genes, while SRL stimulated lipolysis and inhibited expression of lipogenic factors in adipose tissue. The increase in lipolysis with the CsA treatment could be related to the observed up-regulation of HSL gene level (although not statistically significant) and to the increase in its protein expression after 9 weeks. These results correlate with the significantly higher levels of NEFA and TGs observed in serum after 9 weeks with the CsA treatment. Nonetheless, the authors acknowledge that HSL is regulated at the phosphorylation level, which has not been measured in this work. However, similar studies have demonstrated that CsA and SRL increase isoproterenol-stimulated phosphorylation of HSL at Ser552 in human adipocytes and phosphorylation of HSL at Ser563 in 3T3-L1 adipocytes (Chakrabarti *et al.*, 2010; Pereira *et al.*, 2013; Soliman *et al.*, 2010). Furthermore perilipin A, responsible for coating the surface of intracellular lipid droplets, was not significantly changed with either CsA or SRL at these doses, while ATGL, which is responsible for converting triacylglycerol into diacylglycerol, tended to decrease with SRL. Recently, Pereira *et al.* (2013) observed a decrease in perilipin gene expression but no changes in ATGL gene expression with SRL treatment in human adipocytes while Chakrabarti *et al.* (2010) observed a decrease in ATGL gene expression in SRL-treated rats. Differences between the present study and others might be related to the duration and doses of treatments.

No change in gene expression for the transcriptions factors ChREBP and SREBP1 was observed in adipose tissue, although the expression of the SREBP1 gene (both 1a and 1c isoforms) tended to decrease in adipose tissue of for the SRL-treated rats. A significant reduction in SREBP1 gene expression was observed in human adipose tissue treated with SRL by Pereira *et al.* (2013). Moreover, when SRL was administrated in vivo to rats, gene

expression data suggests a reduction in lipid flux and uptake into adipocytes, with down regulation of LPL, CD36, PEPCK and lipin1 as well as PPARγ, known to directly regulate the expression of a large number of genes involved in lipid metabolism, as observed by Houde *et al.* (2010). These results agree with our data for lipin1 and PPARγ, even though we saw no differences in LPL or CD36, which could be dose related. Moreover SRL has also been shown to block the expression of SREBP1 target genes, such as ACC, FAS and SCD1, which also agrees with our results indicating, a role for mTORC1 in fatty acid biosynthesis (Brown *et al.*, 2007; Laplante *et al.*, 2009; Luyimbazi *et al.*, 2010; Peng *et al.*, 2002; Soliman *et al.*, 2010). On the other hand, no changes were observed in ACC1 and FAS gene expression with CsA treatment, in perirenal adipose tissue, in agreement with previous studies by Wu *et al.* (1999) and Jiang *et al.* (2013) and with a recent study where calcineurin knockout mice (*CnAB*$^{-/-}$) developed hyperlipidemia without changes in gene expression of ACC, FAS, SDC1 and SREBP (Suk *et al.*, 2013). However, CsA up-regulated ACC1 and FAS protein expression after 3 weeks, which could be explain by alterations at the transcriptional level. Moreover, CD36 and LPL gene expression tended increase with CsA treatment. These results would suggest an increase in re-esterification of FFA and lipid storage (Wakil *et al.*, 2009), helping to maintain body weight and adipocyte diameter in the CsA treatment similar to the vehicle group.

We also observed a reduction in ACC1 and FAS gene expression in all groups in adipose tissue, after 9 weeks of treatment. This may be age-related, as others have also reported a down-regulation in SREBP-1c, FAS and ACC1 gene expression with age, obesity or diabetes in Zucker fatty (ZF; *fa/fa*) and Zucker diabetic fatty rats (ZDF; *fa/fa*), indicating that these 3 parameters may influence ACC transcript levels (Kreuz *et al.*, 2009; Nogalska *et al.*, 2005).

Furthermore, SRL treatment down-regulated lipin 1 gene expression, another downstream target gene of SREBP1, in adipose tissue. Lipin 1 is responsible for directing lipids to the appropriate storage site in adipose tissue and for decreasing expression of other lipogenic genes, which may cause an impairment in TGs storage (Phan *et al.*, 2005). Lipin1 gene expression has previously been shown to be reduced by SRL (Houde *et al.*, 2010). In addition, SRL treatment has also decrease SCD1 gene and protein expression in breast cancer cell lines and in mice with these tumours (Luyimbazi *et al.*, 2010). As this enzyme is one of the key players in *de novo lipogenic* pathway and is involved in lipid storage and a reduced SCD1 expression in liver and adipose tissue has been shown to promote a reduced

weight gain and increased energy expenditure (Jiang *et al.*, 2005); its down-regulation in adipose tissue in our rat model may explain the decrease weight gain and smaller adipocytes in SRL-treated rats when compared to vehicle or CsA, in agreement with previous studies by Houde *et al.* (2010). This may also be related with the decrease in PPARγ gene expression as a reduction of its activity as a transcription factors involved in lipid droplet formation may compromise its ability to channel fatty acids into adipose tissue (Anderson *et al.*, 2008; Rogue *et al.*, 2010; Staels *et al.*, 2005). Down regulation of these genes by SRL may causes decreased TG storage**.** This reduction, together with the enhanced lipolysis, may potentiate lipid accumulation in tissues, such as muscle and liver (Reue, 2007). In our study, we observed ectopic lipid accumulation in muscle and liver, with SRL treatment mainly after 3 weeks, which is also observed after ablation of rictor in mice (Kumar *et al.*, 2010).

In the liver, although we did not observe significant differences in lipogenic genes or protein expression with CsA or SRL at the doses tested, there was a trend for an increase in ACC1, SREBP1 and DGAT 1 in the CsA group; This might be time-dose related as an effective up regulation has been observed in another study with a higher dose of CsA (Delgado *et al.*, 2012). Moreover an up regulation of hepatic *ACC, FAS and DGAT 1* has been shown to be involved in overproduction of hepatic fatty acids and in the pathogenesis of hypertriglyceridemia in rat suffering from hyperlipidemia associated with the nephrotic syndrome (Zhou *et al.*, 2008).

In addition, while there were no significant differences in IL-6 and TNF-α gene expression with either CsA or SRL treatments in adipose tissue, in liver TNF-α gene expression was significantly increased in the CsA group after 3 weeks. Cytokines such as TNF-α and IL-6 have been shown to play a major role in dyslipidemia in rodents (Hotamisligil *et al.*, 1993), and TNF-α is also known to increase the expression of the LDL receptor in hepatocytes, promoting the accumulation of lipids in the liver and may be one of the mechanisms responsible for the accumulation observed in our results.

Our results are summarized in Table 1 and figure 9. This study shows the effects of relatively short and long term CsA and SRL treatments *in vivo*, using a rat model system. We show some physiological and morphological changes, as well as changes at the protein and gene expression of factors involved in lipid metabolism in both adipose tissue and liver. However, limitations of our study are its descriptive nature and the lack of mechanistic analysis that would help to explain some of the alterations observed in the lipolytic and lipogenic factors, and this should be addressed in future insulin signaling experiments, as the animals in this study were not treated with insulin prior sacrifice. Nonetheless, our model

allows us to demonstrate that CsA stimulated lipolysis without changing lipolytic and lipogenic genes, while SRL stimulated lipolysis and inhibited expression of lipogenic factors in adipose tissue.

Moreover, dysregulation of fatty acid metabolism, a hallmark of immunosuppressive therapy, particularly in adipose tissue, may contribute to the observed elevation of FFA in plasma and to the ectopic fat deposition observed in liver. Further studies on the different pathways and transcription factors involved in lipid metabolism that are affected by immunosuppressive agents, may be beneficial for understanding the pathology of post-transplant dyslipidemia and the design of immunosupressor with less unwanted side effects.

In conclusion, we have shown that both CsA and in particular SRL act at the level of adipose tissue enhancing lipolysis and down-regulating lipogenic genes, which can in part explain the development of dyslipidemia and NODAT during immunosuppressive therapy.

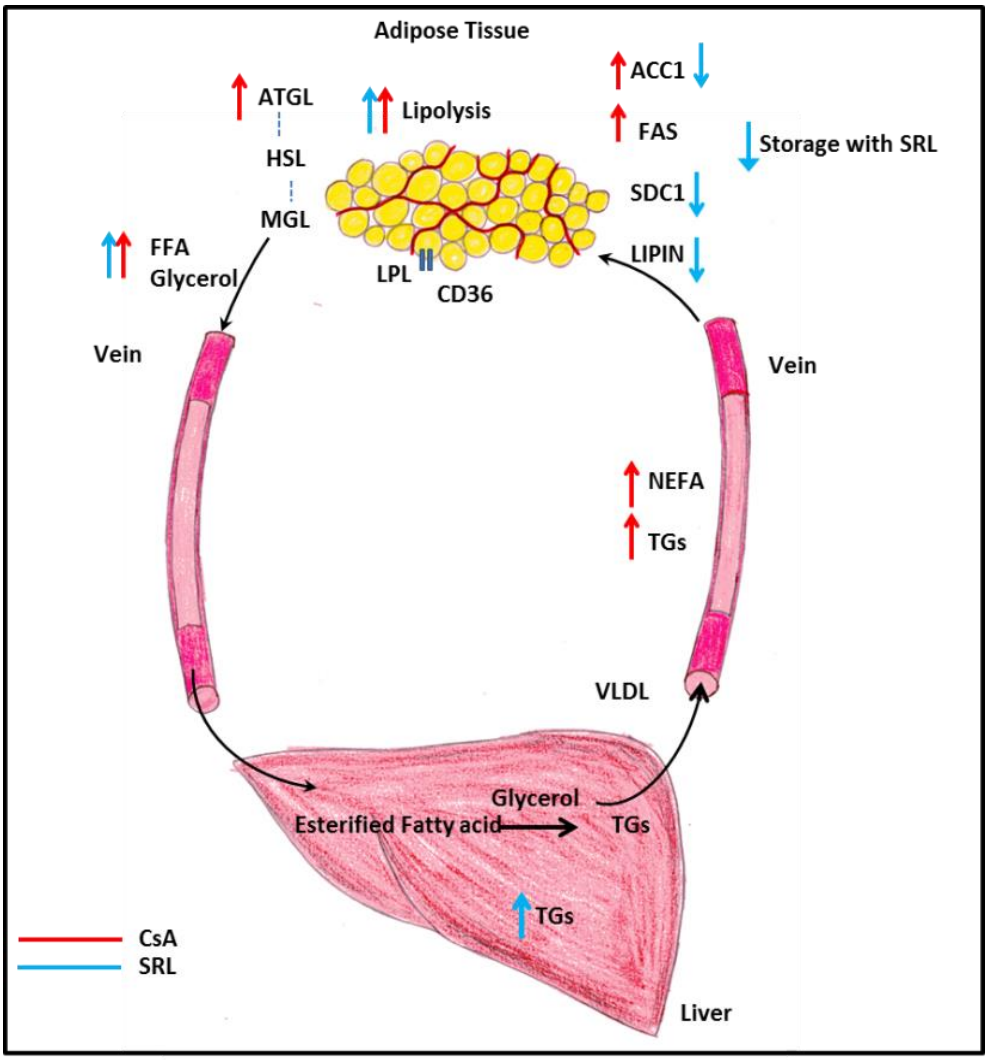


Figure 5.11. Scheme summarizing the effects of CsA and SRL on the crosstalk between liver and adipose tissue. Red arrows correspond to CsA; Blue arrows correspond to SRL. ↑, increase; ↓, decrease.

Chapter 6

NMR-based metabolic profiling of hepatic response to Cyclosporine A

This Chapter comprises the work in preparation by
Lopes PC, Jarak I, Jones JG, Carvalho E

6.1 Introduction

Monitoring the function of a transplanted organ and the effects that immunosuppressive drugs exerted on the recipient body is fundamental for long term graft survival. Metabonomics is a recent instrument available to measure small metabolites that exist in tissue and fluids. Metabolites are the product of metabolism and essential in many biological function and may work as important biomarkers to identify different diseases (Kim *et al.*, 2010). This methodology is promising because changes in the cell biochemistry can be detected in an early phase, well before histologic and pathophysiologic changes occur (Christians *et al.*, 2008). Metabolites might be detected by mass spectroscopy and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (Kim *et al.*, 2010). In rat models and human patients, changes in endogenous metabolites have already been observed by these techniques in blood and urine after CsA administration (Klawitter *et al.*, 2010). Transplantation and the introduction of calcineurin inhibitors, like Cyclosporine A (CsA) have been important to save lives and improve the safety of organ transplants (Heusler *et al.*, 2001). Nonetheless, although short-term outcome of organ survival has improved, long-time survival (< 5 years) is compromised by the development of chronic side effects such as new onset diabetes mellitus after transplantation, dislipidemia or nephrotoxicity (Subramanian *et al.*, 2007). To prevent the toxicity of an immunosuppressive drug, early detection must be made and a dose-

reduction, or regiment switch must be applied (Klawitter *et al.*, 2010). Although metabolomic approach gives insight into the changes of total metabolite concentrations it gives no information on the contributions of different pathways to the total metabolite pool.

CsA is associated with increased risk of glucose intolerance however it is still not clear if its relative diabetogenicity is due to β-cells dysfunction (Oetjen *et al.*, 2003; Polastri *et al.*, 2002), peripheral tissue insulin resistance or both. Moreover, there is limited information on its effects on hepatic insulin resistance. In addition, by reducing the concentration of adipose tissue lipoprotein lipase (Vaziri *et al.*, 2000), it leads to the increased triglyceride plasma levels contributing to insulin resistance.

Hepatic insulin resistance manifests itself in loss of regulation of several important metabolic processes, and results amongst other in decreased glycogen synthesis, increased *de novo* lipogenesis (DNL), and impaired suppression of postprandial hepatic glucose production when portal vain glucose and insulin levels are high. Conventional analysis of postprandial glucose and insulin appearance does not inform on the contributions of absorption and hepatic glucose production to total plasma glucose. These contributions can be resolved by the use of glucose load enriched with stable isotope tracers. Recently, Delgado *et al.* (2012) were able to resolve the contributions of glucose load and endogenously produced glucose (EGP) to plasma glucose in healthy rats treated with CsA during 20 days by applying [U-^{13}C] glucose and $^{2}H_2O$. The analysis revealed impaired suppression of hepatic glucose release by insulin and 60 min after the glucose load the increased EGP contributed to the elevated plasma glucose in CsA-treated rats. Although both gluconeogenesis and glycogenolysis can contribute to EGP, the increased glycogenolysis was found to be responsible for the observed increase of plasma glucose levels. These results combined with the analysis of hepatic glycogen levels suggested that the initial glycogen levels were higher in CsA-treated animals. However, until now the influence of CsA on the contributions of direct and indirect pathways to glycogen synthesis was not determined. Although the expression of several lipogenic enzymes was found to be increased, the contribution of lipogenic deregulation to the onset of CsA induced insulin resistance was not unequivocally confirmed since hepatic DNL was not measured.

Deuterated water $^{2}H_2O$ as a stable metabolic tracer is increasingly used since it has several advantages over other tracers. In addition to the reduced cost it is easily administered, either intraperitoneally or orally, over long periods of time and equilibrates easily with the total body water pool, which avoids inefficient equilibration with the precursor pools, or precursor enrichment gradients. ^{2}H isotopomer distributions in precursor pools and the end

products of the particular pathway provide an insight into the fluxes and the activity of pathway enzymes that participate in the metabolism of a given precursor. Deuterated water has been successfully used in the studies of carbohydrate and lipid metabolism in both animal and human studies.

6.2 Results

6.2.1 Effects of CsA treatment on body weight and adipocyte diameter and weight

Alterations in body weight were monitored during treatment and are shown in fig 6.1A. Wistar rats had a mean weight of 318.8 ± 5.3 g at 8 weeks of age before the beginning of treatments. Although no significant change in body weight were seen between vehicle and CsA group, there was a clear tendency for a loss in weight for CsA group (Fig. 6.1A), as confirmed by the presence of smaller (79,80 ± 3.81 vs. 101.40 ± 6.71, $p<0.05$) and lighter adipocyte cells (0.30 ± 0.04 vs. 0.62 ± 0.12, $p< 0.05$) (Fig. 6.1B).

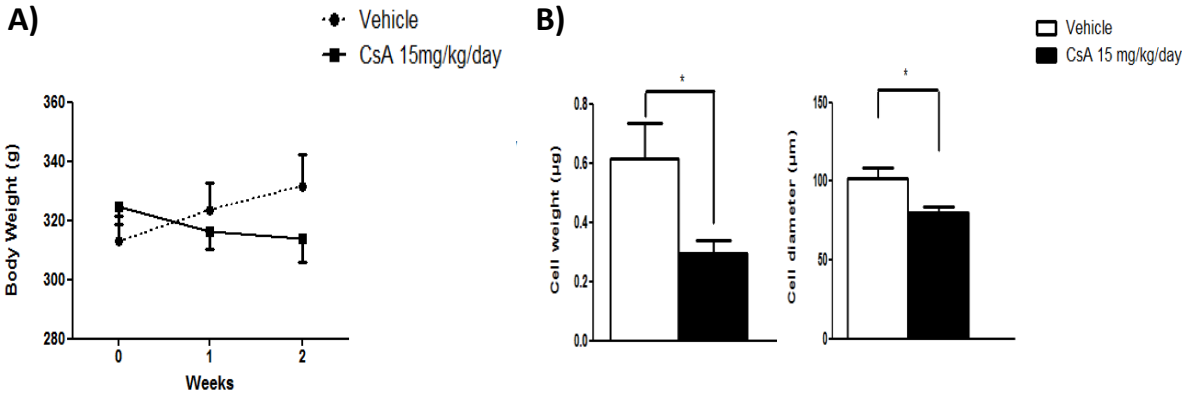


Figure 6.1. Effects of vehicle and CsA on body weight (A), adipocyte diameter and weight (B). Weight was monitored every week until the end of treatments Data are presented as mean ± SEM. Differences between treatments were assessed with one-way ANOVA, and unpaired t-test. * $p<0.05$ vehicle vs. CsA at the same time point.

6.2.2 Effects of CsA on glucose tolerance test

GTT was performed after the 15 days of treatment and fasted glucose was very similar between the two groups. However, 30 minutes after a glucose bolus (2 mg/g, i.p.), the CsA-treated group presented significantly higher glucose levels (383.8 ± 54.6) when compared to the vehicle (240.5 ± 24.3 mg/dl, $p<0.001$). After 60 min, glucose was still significantly higher in CsA group (357.5 ± 37.49 vs. 159.45 ± 14.64, $p<0.001$), never reaching the basal values in the 2 hours of test (Fig 6.2).

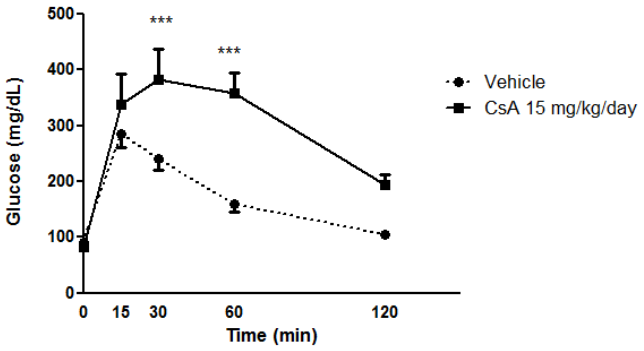


Figure 6.2. Effects of vehicle and CsA on GTT. Glucose levels at time point 0, and after an i.p. injection of glucose (2 mg/kg/body weight) at 15, 30, 60 and 120 min. Data are presented as mean ± SEM. Differences between treatments were assessed with one-way ANOVA, *** $p<0,001$ vehicle vs CsA at the same time point.

6.2.3 Effects of CsA on hepatic glycogen sources

Glycogen ^{2}H enrichment was assessed after its hydrolysis to glucose and subsequent derivatization to monoacetone glucose (MAG). ^{2}H NMR spectra of MAG consists of well resolved singlet peaks that correspond to individual positions of MAG hydrogens (Fig 6.3).

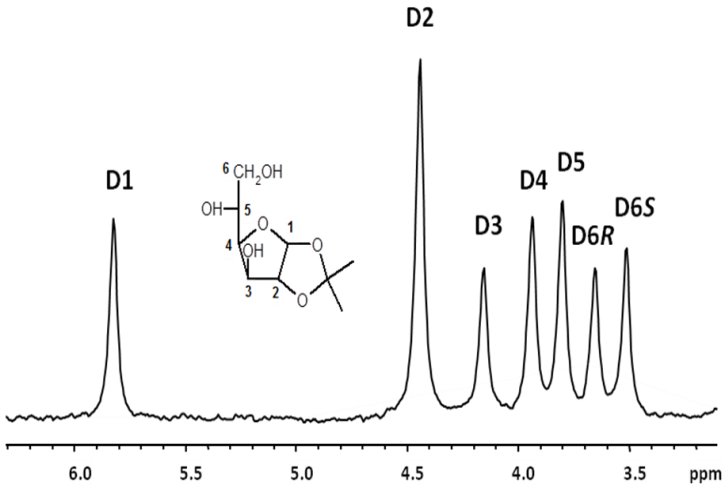


Figure 6.3. ^{2}H NMR spectra of MAG prepared from liver glycogen. D1-D6*R,S* represent the NMR signals of ^{2}H enrichments in positions 1-6 of glycogen glucosyl moieties.

Due to high hepatic concentration of glycogen and high positional ^{2}H enrichments (~1%) high quality spectra (S/N~20) were obtained in relatively short times (2h). Using Eq.1 it was possible to calculate the ^{2}H enrichment on each of MAG carbons (Table 6.1).

Table 6.1. Liver glycogen and body water ^{2}H-enrichment for vehicle and CsA-treated rats

	^{2}H enrichment of body water (BW) and glycogen hydrogens 1 to 6*S*[a]							
Animals	BW	1	2	3	4	5	6*R*	6*S*
Vehicle	2.61	1.06	2.12	0.93	1.15	1.26	1.01	0.91
	2.06	1.19	2.18	0.92	1.20	1.31	0.99	0.91
	1.91	1.24	2.09	0.97	1.25	1.30	1.13	1.02
	2.02	1.02	1.91	0.76	1.00	1.06	0.83	0.77
	1.83	1.00	2.05	0.88	1.15	1.19	1.00	0.92
	1.86	0.99	2.02	0.87	1.08	1.12	0.92	0.85
	2.01	0.98	1.63	0.75	0.94	1.05	0.86	0.75
Mean ± SE	**2.04±0.1**	**1.07±0.004**	**2.00±0.07**	**0.87±0.03**	**1.11±0.04**	**1.18±0.04**	**0.96±0.04**	**0.87±0.03**
CsA-treated	2.20	1.28	2.18	0.94	1.23	1.17	0.84	0.84
	2.14	1.54	2.03	1.13	1.39	1.55	1.28	1.16
	2.00	1.43	2.20	1.10	1.35	1.41	1.16	1.08
	1.81	1.15	2.24	0.94	1.26	1.19	0.98	0.91
	1.96	1.12	1.97	0.92	1.14	1.20	1.00	0.93
	1.95	0.99	2.02	0.87	1.08	1.12	0.92	0.85
	2.09	1.41	2.23	1.09	1.39	1.42	1.19	1.18
	2.22	1.10	1.91	1.00	1.15	1.17	0.89	0.88
Mean ± SE	**2.05±0.05**	**1.25±0.0***	**2.10±0.05**	**1.00±0.03***	**1.25±0.04**	**1.27±0.05**	**1.03±0.06**	**0.98±0.05**

[a] The means and standard errors (SE) for vehicle and CsA-treated groups are shown. *Significantly higher than vehicle value, $P = 0.05$.

Position 2 is the most enriched one and represents glycogen synthesis through both direct and indirect pathways and was found to be the same in both vehicle and CsA-treated animals. Similar enrichment were measured for positions 4 and 6, including position 5 which is characteristic for glycogen synthesized through indirect pathway. Positions 1 and 3 in CsA-treated animals exhibit higher ^{2}H enrichment compared to vehicle animals (1.25% and 1.07%, p=0.042; 1.00% and 0.87%, p=0.016, respectively). Position C-2 was found to be completely exchanged with the body water under the given experimental conditions (H2/BW) and in both groups all the glycogen was derived from glucose-6-phosphate sources. Direct and indirect contributions to hepatic glycogen were estimated for each animal from the ratio of H5/H2 ^{2}H enrichments as described by the equations 2 and 3 (Table 6.2). Direct pathway contributed to the hepatic glycogen with ~ 40% and was found to be the same for both groups of animals. Ratio H3/H5 was used as a measure for transaldolase activity and was significantly higher in CsA-treated animals.

Table 6.2. Liver glycogen synthesis parameters for vehicle and CsA-treated rats

Animals	Hepatic glycogen synthesis parameters[a]			
	H2/BW	Direct pathway (%)	Indirect pathway (%)	H3/H5
Vehicle	0.81	41	59	0.74
	1.06	40	60	0.70
	1.09	28	62	0.75
	0.94	44	56	0.71
	1.12	42	58	0.74
	1.08	44	56	0.77
	0.81	36	64	0.72
Mean ± SE	**0.99±0.05**	**41±0.1**	**59±0.1**	**0.73±0.01**
CsA treated	0.99	46	54	0.8
	0.95	24	76	0.73
	1.10	36	64	0.78
	1.24	47	53	0.79
	1.00	39	61	0.76
	1.03	44	56	0.77
	1.07	37	63	0.77
	0.84	38	62	0.85
Mean ± SE	**1.03±0.04**	**39±0.3**	**61±0.3**	**0.78±0.01***

[a]The means and standard errors (SE) for vehicle and CsA-treated groups are shown; *Significantly higher than vehicle value, $P = 0.05$.

6.2.4 Effects of CsA on de novo lipogenesis contribution to HTG pool

The enrichment of TG methyl hydrogens from $^{2}H_2O$ enriched water provides a measure of *de novo* lipogenic contribution to the total TG hepatic pool while the enrichment of glycerol hydrogen provides information on glycogen cycling. As illustrated in figure 6.4 both ^{1}H and ^{2}H NMR spectra of hepatic TG isolated by Folch extraction provide well resolved TG methyl and glycerol methylene peaks.

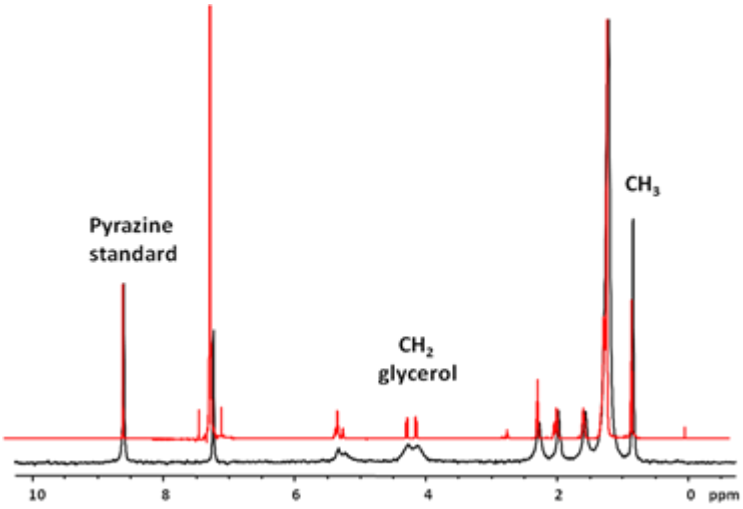


Figure 6.4. ^{1}H and ^{2}H NMR spectra of extracted hepatic TGs.

Concentrations of the labeled and non-labeled TGs were determined by comparison with standard pyrazine and the extent of ^{2}H enrichment of the TG methyl fatty acid and methylene glycerol positions calculated as described by Eq.5 and Eq.6 (Table 6.3).

Table 6.3. ^{2}H enrichments of triglyceride CH_3 (fatty acid) and CH_2 (glycerol) moieties for vehicle and CsA-treated rats

	Vehicle	**CsA**
^{2}H triglyceride CH_3 enrichment (%)	0.18±0.06	0.19±0.09
^{2}H glycerol CH_2 enrichment (%)	0.85±0.12	1.16±0.10

Data are presented as mean ± SEM. Differences between treatments are indicated by * (t-test, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$).

Ratio of obtained positional and body water enrichments gives the fraction of *de novo* lipogenesis and glycerol cycling to hepatic TG pool (Table 6.4). In both groups of animals *de novo* lipogenic contribution to the hepatic TG over 72h was found to be the same (3%). Although tendency towards increased glycerol cycling has been observed in CsA-treated animals (18.52% compared to 13.46% in vehicle group) statistically significant difference was not observed.

Table 6.4. 24 h hepatic triglyceride (HTG) fractional synthetic rates (FSR) (liponeogenic and glyceroneogenic fractions) for vehicle or CsA-treated rats

	Vehicle	**CsA**
HTG liponeogenic FSR (%)	2.79±0.75	3.01±1.25
HTG glyceroneogenic FSR (%)	13.46±1.81	18.52±1.58

Data are presented as mean ± SEM. Differences between treatments are indicated by * (t-test, $^{*}P<0.05$).

CsA treatment considerably increased blood plasma TG concentration (Table 6.5). However the same trend was not observed in the increased amounts of hepatic TG, which exhibited the same amounts of TG per gram of tissue in both groups. Hepatic VLDL production rate was estimated from total TG blood plasma concentrations 4 h after the inhibition of lipoprotein lipase activity with non-ionic detergent Pluronic F-124 (Table 6.5 and Figure 6.5). Despite of the tendency towards increased VLDL in CsA-treated rats (6.07 mg/h/kg compared to 4.46 mg/h/kg in vehicle group) the values were not statistically higher.

Table 6.5. Influence of CsA on hepatic and blood plasma TG content and hepatic VLDL production.

	Vehicle	**CsA**
Blood plasma triglycerides (mg/dl)	94.22±5.79	127.55±10.67*
Hepatic triglycerides (mg/dl)[a]	1.50±0.19	1.62±0.17
Hepatic VLDL production rate (mg/h/kg)[b]	4.46±0.84	6.07±0.64

Data are presented as mean ± SEM; [a] Concentrations expressed per mg of liver tissue; [b] Data are expressed as mg/h/kg using a plasma volume of 35 ml/kg body weight; differences between treatments are indicated by * (t-test, $^{*}P<0.05$).

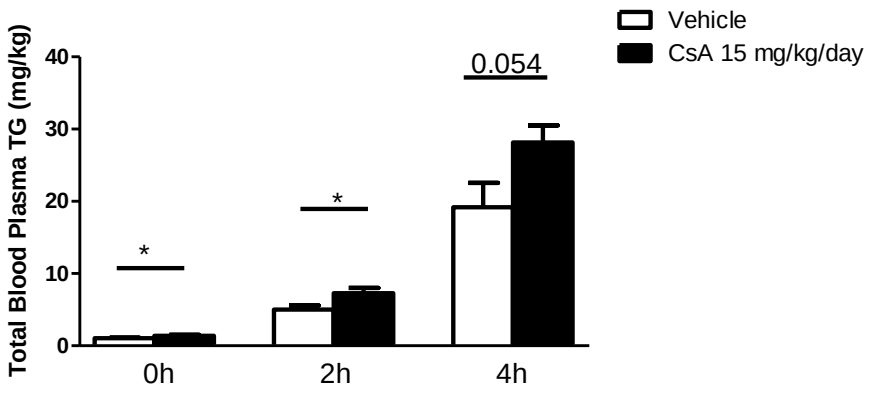


Figure 6.5. Effects of vehicle and CsA on total blood plasma TG concentrations after Pluronic F-124 injection. TG levels increased significantly at time point 0, 2h but not after 4h. Data are presented as mean ± SEM. Differences between treatments were assessed with unpaired t-test, * $p<0,05$ vehicle vs. CsA.

6.3 Discussion

In this *in vivo* study, the dose of 15 mg/kg BW/day of CsA was chosen since it mimics the recommended CsA oral dose in the early post-transplant phase (12 to 15 mg/kg/day) in humans. At these doses and for a short period of time, CsA seems to cause weight loss and an impaired response to GTT. Moreover, no changes were seen in direct/indirect pathway fluxes of hepatic glycogen synthesis and *de novo* lipogenesis between CsA and vehicle. Significant differences were observed in the enrichment of positions 1, 3, and 4 of glycogen labeling, suggesting that there were some alterations in hydrogen exchange reactions between sugar phosphate metabolites and body water, but these do not directly translate to changes in glycogen synthesis fluxes.

CsA at this dose seems to promote a weight loss compared with the vehicle group, in accordance with results by Böhmer *et al.* (2010), which was clearly seen in the decreased adipocyte weight and diameter. Moreover, during the GTT performed, CsA presented significantly higher glucose levels after 30 and 60 min of the glucose bolus and consequently a slower glucose excursion rate, reflecting a higher hyperglycemic/diabetogenic response. This could be the result of the inhibitory effects of CsA on β-cell survival, and therefore, causing a decrease in insulin secretion as observed by others (Øzbay *et al.*, 2012; Redmon *et al.*, 1996 ; Uchizono *et al.*, 2004).

Incorporation of ^{3}H or ^{2}H label from enriched water into glucose allows not only the estimation of rates of glucose and glycogen synthesis but gives valuable insight into the substrates from which they are formed. The NMR analysis of MAG derivative described by Delgado *et al.* (2012) is based on comparison of positional enrichments of MAG with that

of body water which is the precursor of label. Body water enrichment is easily determined by analyzing blood plasma, while MAG derivative enables good resolution of glucose NMR signals. As described previously (Delgado, unpublished data) after the intraperitoneal bolus of $^{2}H_2O$ total body water ^{2}H enrichment reaches isotopic steady state within 10 minutes. After the administration of initial bolus of $^{2}H_2O$ the body water enrichment is maintained by ^{2}H enriched drinking water. Due to the short wash-in period of the tracer the enrichment of the body water measured at the end of the experiment (72 h) represents the precursor ^{2}H enrichment over the entire experiment period. The final body water enrichment (2% for both CsA-treated and vehicle group) was significantly lower than the aimed enrichment (5%). However as described by Murphy (2006), measured body water enrichment is expected to be lower than the aimed one due to dilution of enriched body water from water that originates from food or respiration and under the experimental conditions of prolonged $^{2}H_2O$ exposure (72 h) these effects on tracer dilution were found to be considerable.

As described by Rognstad *et al.* (1974) in their experiment with ^{3}HHO the pattern of tritium incorporated into glucose depends on the type of glucogenic substrate. Thus, the incorporation of tritium ^{3}H or deuterium ^{2}H from labeled water can provide valuable information not only about gluconeogenic substrates but also about the enzyme activities or futile cycles.

As observed in ^{3}HHO experiments when lactate or pyruvate is the substrate, glucose hydrogens at the position C-6 are extensively labeled. This labeling can be traced back to the incorporation of ^{3}H into two positions of C-3 of malate (Fig. 6.6). Position C-3*R* of malate will be labeled as the result of reversible fumarase reaction. Cycling through the Krebs cycle will introduce ^{2}H from the labeled medium in the positions C-2 and C-3*S* of malate and due to the symmetricity of fumarate these labeling will be randomized by fumarase. Additional enrichment in positions C-2 and C-3 of malate is possible through the labeled acetyl-CoA that enters citric acid cycle. Methyl hydrogens of acetyl-CoA originate from C-3 hydrogens of pyruvate that are enriched in the pyruvate futile cycle (pyruvate-oxaloacetate-phosphoenolpyruvate-pyruvate) through pyruvate kinase catalysed reaction (Fig. 6.7). In turn, labeling in position C-2 of malate is the source of labeling for NADH that is used as reducing equivalent in the reactions of gluconeogenesis and glyceroneogenesis. Mitochondrial NADH can also be labeled from the medium through the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase reactions (Fig. 6.6 and 6.7). Alternative source of NADH (and NADPH needed for FA synthesis) labeling is the glutamate dehydrogenase reaction since labeling in C-2 position of glutamate is possible due to

transaminase exchange. The other possibility for the labeling to appear in the C-6 position of glucose is via labeling the position C-1*R* of unlabeled fructose-6-phosphate in glucose-phosphate isomerase exchange reaction between glucose-6-phosphate and fructose-6-phosphate during glucose futile cycling (Fig. 6.8). Through subsequent aldolase and triose-phosphate isomerase exchange reactions glyceraldehyde-3-phosphate will be labeled in C-3 position. As a result of described futile glucose cycling glucose will be labeled in only one of two C-6 glucose positions. Labeling in position C-1 of glucose is related to the labeling of C-3*R* position of malate and was found to be about the same as the average enrichment incorporation in the position C-6 in $^{3}H_2O$ experiment. This would suggest that, at least in the case of isolated rat liver parenchymal cells, the most significant incorporation mechanisms are similar. However, the NMR based analysis of glycogen derived MAG reveals significant difference between C-1 and C-6 enrichment, as well as significant increase of enrichment in C-1 position of CsA-treated rats compared to the vehicle group. The labeling of both C-6 hydrogens in both groups was the same. Labeling in position C-4 is introduced through glyceraldehyde-phosphate dehydrogenase catalysed reduction of 1,3-biphosphoglycerate (Fig. 6.6). Reversible reactions between glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate catalysed by triosephosphate isomerase and aldolase will introduce further labeling into positions C-3 of dihydroxyacetone-phosphate and C-1 and C-2 of glyceraldehyde-3-phosphate and thus into positions C-3, C-4 and C-5 of glucose (Fig. 6.8). Although the enrichment on C-4 and C-5 of glucose was found to be the same within both groups of animals, that on position C-3 was considerably smaller. Enrichment on position C-3 was found to be significantly smaller in vehicle group than in the CsA-treated group. However, other processes than the above described ones, like transaldolase exchange or primary kinetic isotope effect of triosephosphate-isomerase can influence the enrichment on position C-3 and increase C-5/C-3 enrichment ratio(Bock *et al.*, 2008). Enolase responsible for the reversible conversion of 2-phosphoglycerate to PEP will result in additional labeling of position C-2 of glyceraldehyde-3-phosphate and C-5 of glucose (Figure 6.6).

Labeling at C-2 of glucose occurs for all the glucose-6-phosphate sources that contribute to the hepatic glycogen and is the result of glucose-6-phosphate isomerase activity. It is equal to the ratio of the enrichment in position H2 and BW enrichment. Whereas glucose contributing to glycogen through the direct pathway will be labeled only in the position C-2, glucose contributing through indirect pathway will be labeled in position C-5 as well due to the label exchange at the triose-phosphate level. Therefore, the ratio of C-5/C-2 enrichment of glucose derived from glycogen can be used to estimate the fraction of

glycogen derived from gluconeogenesis. Contributions of direct and indirect pathways are the same for both vehicle and CsA-treated animals. Although the enrichments of C-3 and C-5 of glucose basically have the same origins, labeling on position C-3 can be influenced by transaldolase exchange. The ratio of H3/H5 enrichment was therefore tentatively used as the measure of transaldolase activity. Results of the NMR MAG analysis suggest that transaldolase activity was decreased due to CsA treatment.

Changes in relative contribution of DNL to fatty acid pool provide interesting information about processes such as TG assembly and production, and has been studied with various stable isotope traces including $^{2}H_2O$. However, in stable isotope studies, the accurate determination of the true precursor enrichment is difficult when that particular precursor is not biochemically available, which is the case with acetyl-CoA, the basic building block in fatty acid synthesis. Given the complex sources of fatty acid hydrogens each precursor pool can potentially have different enrichments and quantitative determination of DNL is limited by certain assumptions. In mass spectrometry (MS) based measurements of ^{2}H enrichment, mass isotopomer distribution analysis (MIDA) based on mathematical principles of combinatorial probabilities was developed in order to determine the true precursor enrichment. By comparing the measured isotope pattern of a given metabolite with expected statistical distribution of possible enrichment sites *n* it is possible to determine the isotopic enrichment of the precursor pool (Hellerstein *et al.*, 1999). The use of MIDA in the analysis of fatty acid synthetic rates provided the *n* values based on composite contributions of different hydrogen pools. Values of *n* were found to be strongly dependent upon conditions and the tissue being examined.

Recently,Delgado *et al.* (2009) proposed a novel method of DNL quantification based on *ex vivo* ^{2}H NMR analysis of triglyceride ^{2}H enrichment from $^{2}H_2O$. During DNL terminal methyl hydrogens are derived directly from acetyl-CoA (Figure 9) and do not participate in the processes of hydrogen exchange that occur during the FA chain elongation. The proposed method is based on the assumption that pyruvate is the main source of lipogenic acetyl-CoA and that the exchange of pyruvate CH_3 protons with $^{2}H_2O$ is essentially complete. Under these conditions acetyl-CoA ^{2}H enrichment is considered to be the same as the body water enrichment and can be used as the true precursor enrichment. However, the DNL contribution will by underestimated to the extent that the methyl hydrogens are not fully exchanged with those of body water (Rognstad *et al.*, 1974; Zhang *et al.*, 2006). The other assumption the method is based on is the fact that the hepatic TG pool is completely turner over during the $^{2}H_2O$ exposure experiment. Unless the turn-over is complete

underestimation of DNL occurs. The complete turn-over in healthy humans has been estimated to be less than 48 h (Vedala *et al.*, 2006) and taking into account faster basal metabolism in rats it is assumed that the total TG pool in rats will be completely turned over during 72 h of ^2H_2O exposure.

Since in the ^2H NMR spectrum methyl signals of palmitoyl (C16) and stearoyl (C18) chains co-resonate the measured methyl group enrichment represents the contribution of C16 and C18 acyl moieties to DNL (Fig.6.9). Although part of stearic acid present in the extracted TG is derived by palmitoyl chain elongation the resulting enrichment is introduced in the carboxyl end of the chain and does not contribute to stearic acid derived from DNL. CsA treatment did not influence FSR of TG fatty acids. After 72 h of ^2H_2O exposure, about 3% of total hepatic TG pool was derived from *de novo* lipogenesis in both groups. Enrichment of C-1and C-3 positions of TG glycerol was much higher and indicates that hepatic TG undergo hydrolysis and considerable glycogen futile cycling. However, although there is a tendency of higher glycerol FSR in CsA treated rats, no statistically significant difference was observed (Table 6.4).

To test the influence of CsA treatment on the development of hypertriglyceremia, the production of VLDL, the carrier of hepatic TG in plasma, was measured. Under the fasting conditions where the VLDL is the only source of plasma TG, the rate of TG accumulation in blood is the indicator for hepatic VLDL production rate. The property of non-ionic detergent Pluronic F-124 to inhibit TG hydrolysis by lipoprotein lipase and which results in a progressive increase in the concentration of TG in the blood was used to determine the rate of VLDL synthesis (Millar, 2005). Blood plasma TG levels were significantly higher in CsA group of rats before the detergent injection. Although the total hepatic TGs have not been determined, no difference was observed in TG concentrations per gram of hepatic tissue. Hepatic VLDL rate was determined 4 h after the detergent injection. Although the total plasma TG concentration determined 2 h after the injection was higher in the CsA-treated animals, after 4 h no significant difference was observed despite the observed trend toward higher VLDL production in CsA treated animals. However, in some animals there was no observed increase in plasma TG after the detergent injection and the *n* should be increased in order to obtain more reliable results.

Taken together these results indicate that CsA at this dose and time of treatment affects body weight and glucose tolerance, but not glycogen synthesis or *de novo* lipogenesis.

Suplementary data

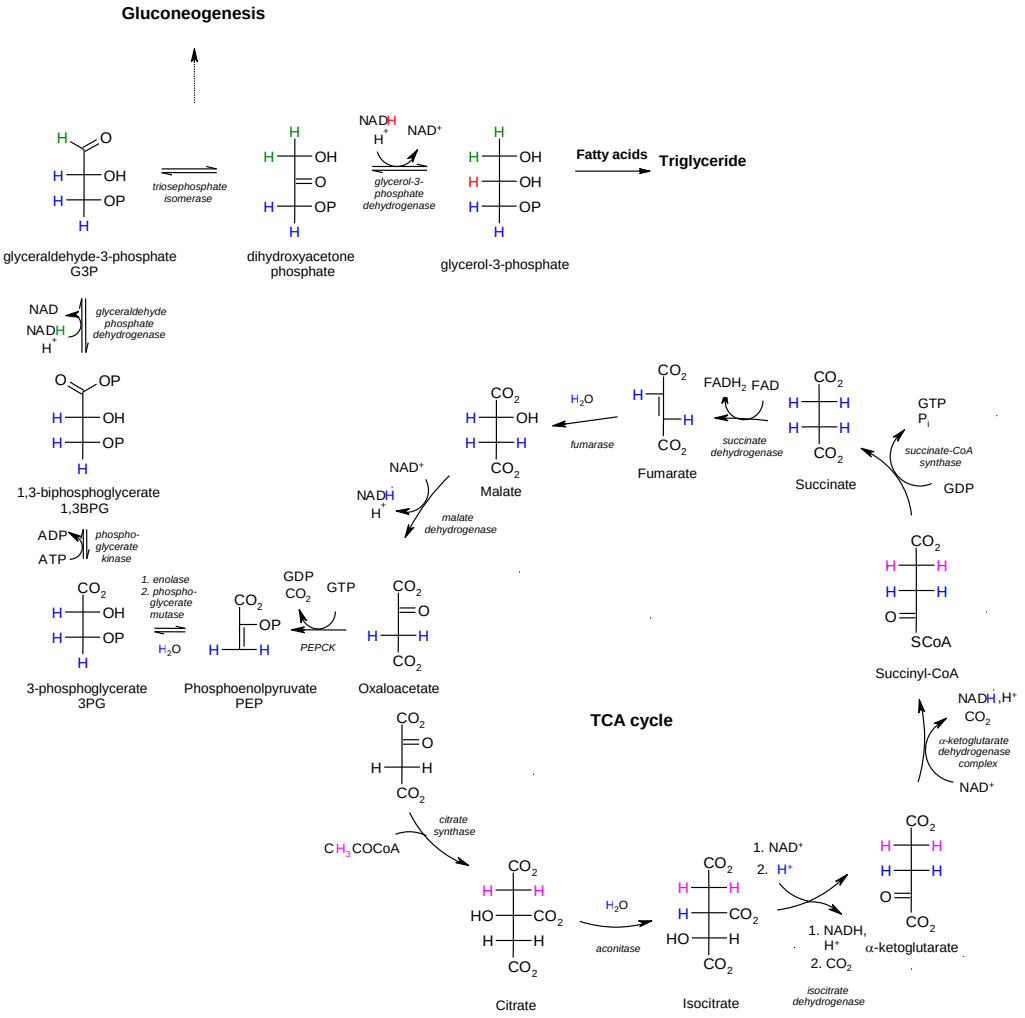


Figure 6.6. Incorporation of ^{2}H from deuterated water into triosephosphate precursors and glycerol: H denotes potential incorporation sites of ^{2}H from gluconeogenic and glyceroneogenic pathways; H and H denotes potential incorporation of ^{2}H into triosephosphate pool; H denotes ^{2}H that enter the Krebs cycle via labeled acetyl-CoA (at the level of succinate they are no longer distinguishable from the medium ^{2}H that enrich the Krebs cycle intermediates due to the molecule symmetry).

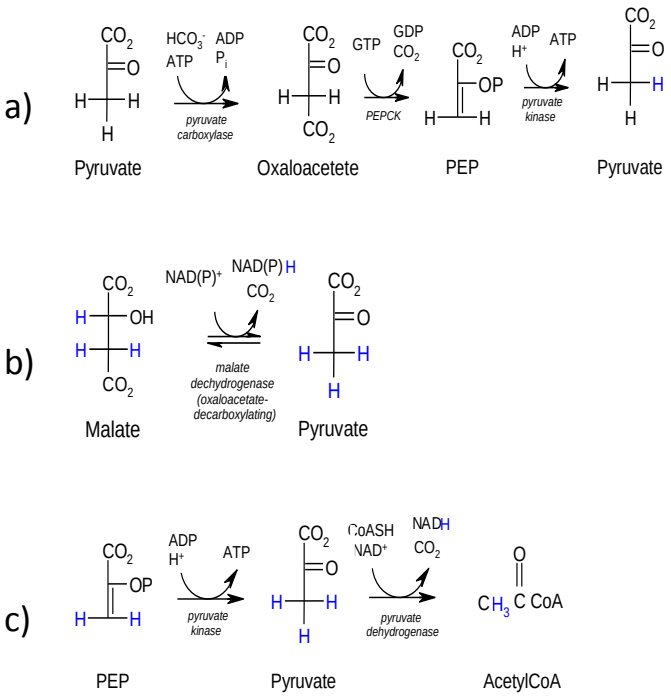


Figure 6.7. Possible mechanisms of ^{2}H incorporation into acetyl-CoA: methyl hydrogens enriched a) during pyruvate futile cycling; b) reversible malate dehydrogenase (oxaloacetate-decarboxylating) catalysed reaction; c) from PEP enriched through the krebs cycle.

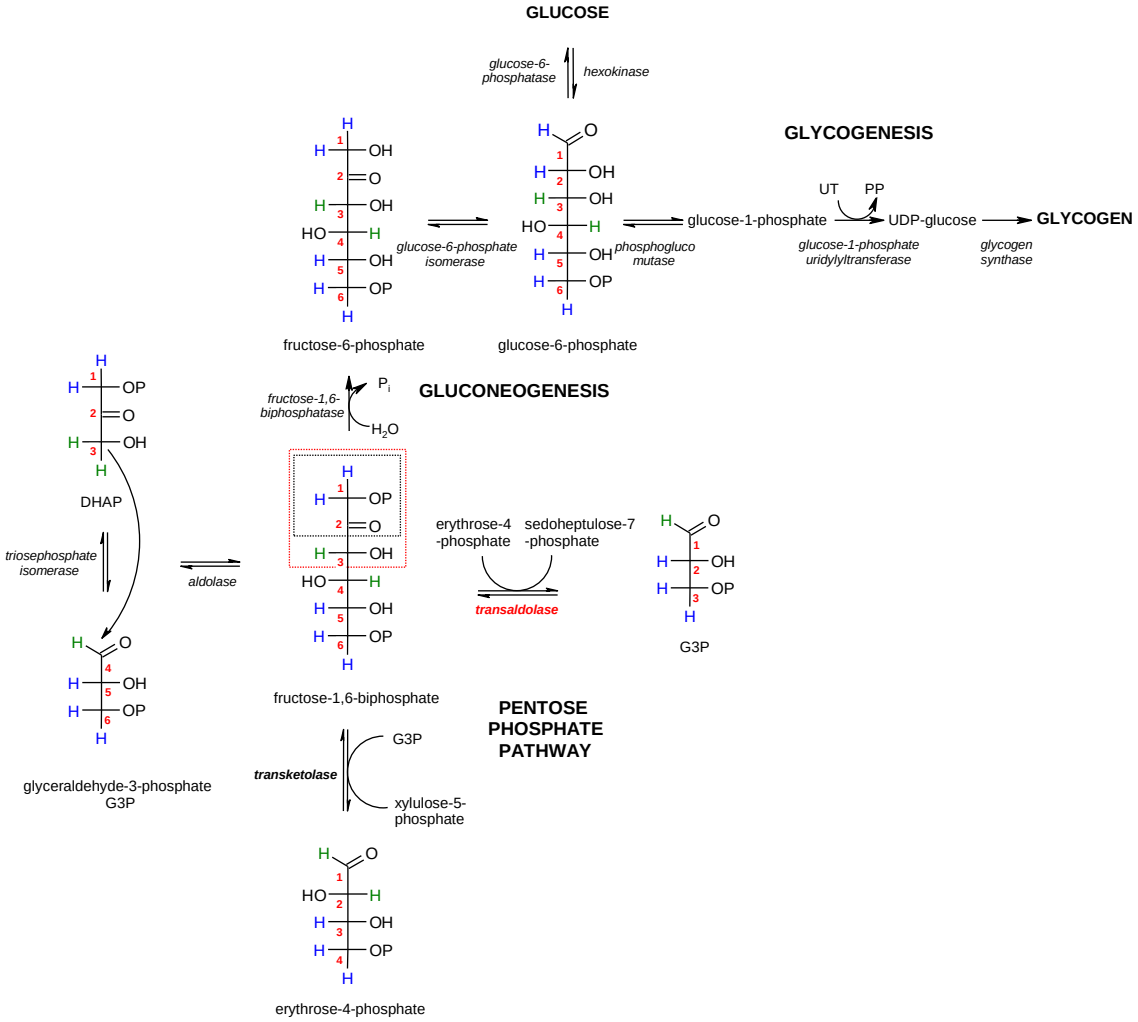


Figure 6.8. Incorporation of ^{2}H into glucose molecule through the labeled substrates.

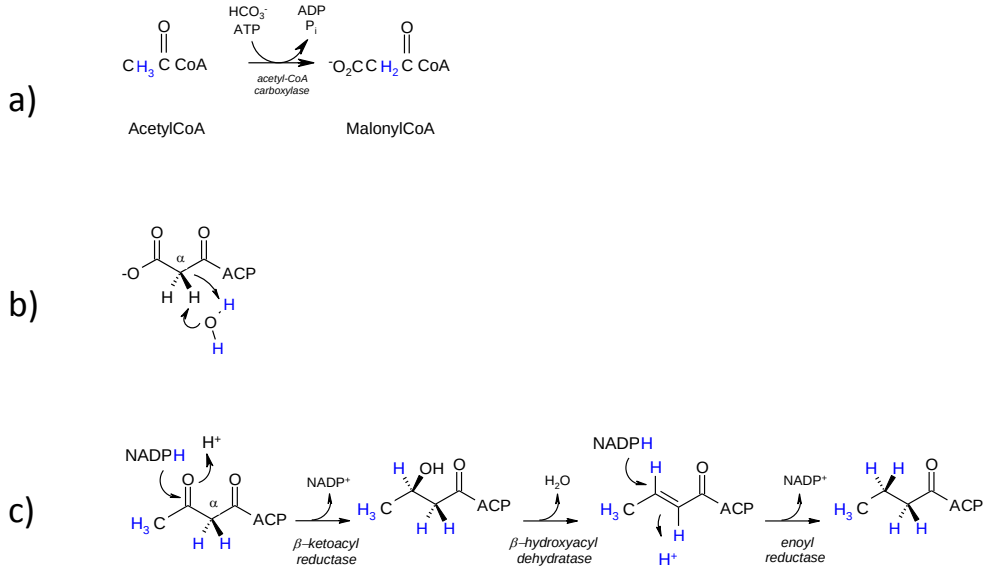


Figure 6.9. Incorporation of ^{2}H into glycerol and fatty acid components of triglycerides: a) synthesis of labeled malonate used in FA chain elongation; b) potential malonate enrichment through the acid methylene hydrogen-medium exchange; c) ^{2}H enrichment of FA even- and odd-C positions during chain elongation.

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Chapter 7

General Discussion/Conclusions

Future Perspectives

In recent years, organ transplant has been a medical procedure more frequent and necessary, as our life expectancy increases and our body suffers the consequence of aging and the development of metabolic diseases. Immunosuppressive agents are essential in order to avoid allograft rejection, increasing the success rate of transplantation. Although they have been used for many years, their molecular effects have not been fully understood. Basic research is important in order to identify the different molecular targets affected by these agents, so pharmaceutical companies can employ their resources in developing better agents with fewer side effects.

In this work, we focused on the effects of two particular agents, CsA, a calcineurin inhibitor and cornerstone of immunosuppressive therapy, and SRL, an mTOR inhibitor and a promising agent. The global effects of these agents on glucose and lipid metabolism are well known, including the development of NODAT and dyslipidemia, affecting the quality of life of the patients and the success of the transplant.

The first aim of this thesis was to explore the effects of therapeutic doses of CsA and SRL on glucose homeostasis and glucose uptake in peripheral insulin sensitive tissues. The results presented in chapter 3 and 4, show that pharmacological doses of these agents promote glucose intolerance and insulin resistance. When taken for a longer period of time (9 weeks), CsA promoted fasting hyperglycemia and decreased insulin levels, which might be the result of a possible deficiency in insulin secretion/production. CsA-treated animals also had increased adipocyte diameter and weight, parameters that contributes to insulin

resistance as suggested by the fact that in large fat cells, insulin stimulation does not increase the amount of GLUT4 in the plasma membrane (Franck *et al.*, 2007).

On the other hand, and also after 9 weeks, SRL-treated rats maintained the normoglycemia but presented higher levels of insulin, suggesting insulin resistance. This was confirmed by higher value of HOMA-IR than the vehicle group. In our rat model, after 3 and 9 weeks of treatment, the CsA group presented an impaired response to a glucose challenge when compared to either the vehicle or SRL-treated groups, presenting higher values of glucose at the different time point after the glucose bolus, during a GTT. On the other hand, the glucose excursion curve of the SRL-treated group was also impaired, as the recovery kinetics of the blood glucose levels was slower after 3 and 9 weeks. Moreover, the ITT showed clearly that the glucose levels in the CsA-treated group were higher, and remained elevated for a longer period. This was also observed in the SRL group, as the glucose values reached higher concentration than the vehicle group. Since SRL is considered to be less nephrotoxic than CsA, and is a valid alternative to calcineurin inhibitors therapy during the maintenance phase, we assessed the glucose clearance rate in the urine. This parameter tended to be increased in the SRL-treated group, suggesting higher glucose levels in the urine than the renal tubule can absorb.

Since *in vivo* treatment with CsA and SRL caused hyperglycemia and insulinemia in the animals, we evaluated whether these agents impaired adipocytes glucose uptake in both *ex vivo* and *in vivo* settings. *Ex vivo* treatment reduced insulin-stimulated glucose uptake in rat isolated adipocytes in a concentration-dependent manner, as already observed in isolated human adipocyte (Pereira *et al*., 2012; Pereira *et al*., unpublished data) and similar to glucocorticoids effects (Lundgren *et al.*, 2004). In addition, *in vivo* treatment for 3 and 9 weeks with either CsA or SRL at therapeutic doses also reduced insulin-stimulated glucose uptake in isolated adipocytes. In order to further understand the impairment in insulin-stimulated uptake, we closely studied glucose metabolism, namely gluconeogenesis and insulin signaling (chapter 4).

Insulin is an important hormone that promotes energy storage and utilization of glucose through glucose uptake, mediated by glucose transporters. After 3 weeks of treatment with SRL, IRS-1 protein level was increased in liver, muscle and adipose tissue, while phosphorylation of IRS-1 at Tyr612 was decreased. Moreover, phosphorylation of the insulin receptor at Tyr1146 residue and Akt phosphorylation at Ser473 and Thr308 residues was decreased in liver and adipose tissue. None of the key factors involved in the insulin signaling were affected by CsA treatment, as also observed in isolated human adipocyte

(Pereira *et al*. unpublished data). Phosphorylation of AS160, an important substrate of Akt that activates translocation of glucose transporters to the plasma membrane, was also decreased in SRL group. Although no changes were observed in GLUT4 protein expression, we cannot exclude the possibility that GLUT4 translocation might have been affected by these drugs, but we did not perform this assay in this study. Moreover, the mTOR pathway was effectively blocked by SRL, as evidenced by the lack of phosphorylation p70S6K at thr421/424 in the three tissues, which is directly activated by mTORC1, the complex sensitive to SRL.

In our model, after only 3 weeks of treatment with CsA the G6Pase protein level was clearly increased and accompanied with a trend for an increase in protein expression of PEPCK, and the transcriptional factors PGC1-α and FOXO1, confirming an enhanced hepatic gluconeogenesis. NODAT has been associated with dysfunctional hepatic gluconeogenesis and inefficiency of insulin to inhibit this pathway. On the other hand, GK protein level, the enzyme responsible for producing glucose-6-phosphate, and consequently glycogen, was decreased in SRL-treated group, suggesting that the glycogen production was decreased in this group. Moreover, PTP1B protein expression, a negative regulator of insulin signaling and also associated with insulin resistance, was increased in the CsA group in the liver. To our knowledge this is the first report to show an effect of CsA on PTP1B gene and protein expression. This increase in PTP1B protein expression may be linked to an increase in insulin resistance and gluconeogenesis observed after CsA treatment.

Insulin sensitivity may also be affected by intracellular lipid accumulation in other tissue than the adipose depot, through impairment of the IRS1-PI3K-AKT signaling pathways. In fact, after 3 weeks of treatment with SRL, there was accumulation of TGs in liver and muscle (chapter 5). Down-regulation of key genes involved in lipid metabolism by SRL in adipose tissue, may contribute to reduced TG storage in these depot. This reduction, together with enhanced lipolysis in SRL-treated animals, may potentiate lipid accumulation in tissues such as muscle and liver. As a matter of fact, SRL decreased the expression of several genes involved in lipogenesis in adipose tissue, including ACC1, lipin 1, SDC1 and PPAR-γ. Although, we did not observe changes in gene expression for the transcriptions factors ChREBP and SREBP1, the expression of the SREBP1 gene tended to decrease for the SRL-treated rats in adipose tissue. Furthermore, SRL treatment down-regulated gene expression of lipin 1 and SCD1, downstream target gene of SREBP1, responsible for the appropriate lipid storage in adipose tissue and *de novo* lipogenic pathway. A reduced SCD1 and lipin 1 expression in adipose tissue might be responsible for the decreased weight gain

and smaller adipocytes of SRL-treated rats when compared to vehicle and CsA. On the other hand, levels of NEFA and TGs are higher in the CsA-treated group after 9 weeks, which can be related with the increase in isoproterenol-stimulated lipolysis and HSL protein expression. In addition, while there were no differences in IL-6 and TNF-α gene expression for either CsA or SRL treatment in adipose tissue, the liver TNF-α gene expression was significantly increased in the CsA group after 3 weeks of treatment. Cytokines, such as TNF-α and IL-6, play a major role in dyslipidemia in rodents and TNF-α is also known to increase the expression of the LDL receptor in hepatocytes. These factors contribute to the lipid accumulation in the liver that we observed. However, with these doses of immunosuppressive agents no significant differences in lipogenic genes were observed in liver.

In chapter 6, we used ^{2}H NMR with the incorporation of ^{2}H from deuterated water ($^{2}H_2O$) to quantify the ^{2}H-enrichment of glucose, glycogen and TG after administration of a higher dose of CsA in vivo for 15 days. We determined that CsA at this dose decrease body weight as well as glucose tolerance, indicated by the higher glucose values observed during a GTT. Although significant differences were determined in the enrichment of positions 1, 3, and 4 of glycogen labelling, no significant changes were observed in direct/indirect pathway fluxes of hepatic glycogen synthesis between CsA and vehicle. Moreover, no differences in *de novo* lipogenesis were found under these conditions. We used non-ionic detergent Pluronic F-124 to inhibit TG hydrolysis by suppressing lipoprotein lipase. Blood plasma TG levels were significantly higher in the CsA-treated group of rats before the detergent injection, nonetheless after 4 h no significant difference was observed despite a trend toward higher VLDL production in CsA-treated animals compared to vehicle. In addition, no differences were seen in hepatic TG concentrations per gram of hepatic tissue. No such measurements have been performed for SRL.

There is an important complex cross-talk between liver and adipose tissue, regulating the contribution of adipose tissue as a storage site and liver to the *de novo* synthesis of fatty acids. Furthermore, skeletal muscle is the main tissue responsible for insulin-dependent glucose uptake by which normal whole body glycaemia levels are maintained. In this work, we demonstrated that CsA at therapeutic doses affects glucose metabolism, by increasing gluconeogenesis in liver, while SRL treatment affects insulin signaling in liver, muscle and adipose tissue; thus affecting glucose tolerance and glucose uptake. Moreover, CsA and in particular SRL, act at the level of adipose tissue enhancing lipolysis and down-regulating key lipogenic genes, impairing lipid metabolism. The deregulation of this balance led to

ectopic deposition of lipids in muscle and liver. These effects might be at the origin of the development of insulin resistance and dyslipidemia observed in patients during immunosuppressive therapy, and caution is required when choosing the therapy to apply to patients.

In future studies, more insulin-signaling/mechanistic studies will be needed to better understand the effects of these agents on the molecular targets studied. In addition to the effects on total amount of phosphorylation of some key factors involved on glucose and lipid metabolism presented on this thesis, it would be important to assess differences in enzymatic activity as well as impairments in phosphorylation levels of other proteins linked to glucose metabolism, namely GK and PTP1B and to lipid metabolism, HSL. Moreover, experiments of GLUT4 translocation would be of importance to understand how CsA and SRL affect the translocation of GLUT4. It would also be important to use NMR technology to determine differences between metabolites of glucose and lipid metabolism in samples for the animals treated *in vivo* with CsA and SRL at either shorter or longer time points. Moreover, as regimens to minimize the use of calcineurin inhibitors when combined to mTOR inhibitors and the right time to apply the change of therapy is a field under exploration, it would be important to study if an early or a late conversion would produce a benefit to avoid the development of dyslipidemia and NODAT after immunosuppressive therapy.

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