**Universidade do Minho**
Escola de Ciências

Carla Manuela Mendes de Sá

# Natural compounds in the control of diabetes associated dyslipidemia: effects in the liver and small intestine

Abril de 2012

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# Natural compounds in the control of diabetes associated dyslipidemia: effects in the liver and small intestine

Tese de Doutoramento em Ciências
Especialidade em Biologia

Trabalho realizado sob a orientação da
**Professora Doutora Cristina Pereira-Wilson**

Abril de 2012

À memória de A. Matias

*"Num deserto sem água*
*Numa noite sem lua*
*Num país sem nome*
*Ou numa terra nua*
*Por maior que seja o desespero*
*Nenhuma ausência é mais funda do que a tua."*

*Ausência,*
*Sophia de Mello Breyner*

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To those who taught me to *always face life with a smile*… I will never forget you. Thanks for being with me and never let me fall apart.

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(To your family for accepting me as I am).

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*Words can not express what you mean to me* ...since to me, you mean the world.

*Take care Champion...*

**Natural compounds in the control of diabetes associated dyslipidemia: effects in the liver and small intestine**

## Abstract

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by elevated blood glucose levels (hyperglycaemia) as a result of defects in both insulin secretion and/or action. T2DM represents a major cardiovascular risk since, in addition to hyperglycaemia, diabetic patients frequently present an abnormal plasma lipid profile (diabetic dyslipidemia). Since the disease is gaining epidemic proportions, novel or complementary therapeutic interventions that open new paths for addressing hyperglycaemia and dyslipidemia may offer promising results to the approaches presently available. The present thesis aimed to study the effects of two species of the genus *Salvia* (*S. officinalis* and *S. fruticosa*) in preventing diabetes and its associated complications. In addition, this work also intended to identify potential antidiabetic and lipid-lowering natural compounds from these medicinal plants, and to characterize their mechanisms of action in target organs relevant for diabetes progression (the liver and the small intestine).

In order to study the antidiabetic potential of *Salvia officinalis* water extract (prepared as a tea), a pilot trial was performed in healthy human volunteers, selected from an age group considered at risk to develop diabetes (*chapter 2*). This tea consumption ameliorated the lipid profile and increased the antioxidant defences of the volunteers, without causing hypoglycaemia and/or hepatotoxicity. These data indicate that *S. officinalis* tea may offer compounds with beneficial properties on the prevention of cardiovascular diseases (CVDs), the major cause of morbidity and mortality in diabetic patients.

In a subsequent study, using dietary carbohydrate manipulation, the effects of the aqueous extracts of *Salvia fruticosa* (SFT) and rosmarinic acid (RA), the major phenolic compound of *S. officinalis* and *S. fruticosa* extracts, in several pathways of the lipid metabolism were evaluated (*chapter 3*). While the low carbohydrate (Lc) diet showed to reduce plasma cholesterol levels, SFT treatment during the four days of the reintroduction of the normal (referred as the high carbohydrate, [Hc]) diet showed to increase HDL cholesterol levels. Both SFT and RA treatments showed a potential to regulate the expression of transcription factors that modulate several lipogenic and

cholesterolgenic enzymes. In addition, RA drinking may be useful in modulating the hepatic fatty acid oxidation and preventing the carbohydrate-induced raise of intestinal cholesterol uptake.

*Chapter 4* is dedicated to the study of the effects of luteolin-7-glucoside (L7G), a dietary flavonoid also present in *S. officinalis* extract, on lipid metabolism in healthy rats. The results indicated that, like RA (*chapter 3*), L7G stimulated the expression of genes involved in fatty acid oxidation. In addition, L7G showed to downregulate the expression of the rate-limiting enzyme of the cholesterol biosynthetic pathway and to potentially repress the sterol regulatory element-binding protein-1 (SREBP-1) activity.

Finally, a study was designed to evaluate the effects of glucose and RA in butyrate (NaBu)-differentiated HT-29 and spontaneously differentiated Caco-2 cells, frequently deemed good *in vitro* models of the intestinal epithelium (*chapter 5*). The results showed that the cellular growth and differentiation status of both cell lines were differently affected by glucose. A subcellular fractionation procedure revealed an increase of the sodium-glucose cotransporter 1 (SGLT1) protein at the apical membrane (BBM) and a raise on the facilitated glucose transporter 2 (GLUT2) in the remaining intracellular membrane fraction (including the basolateral membrane, [BLM]) of NaBu-differentiated HT-29 cells. RA did not affect SGLT1 expression in NaBu-differentiated HT-29 cells. This evidence disagrees with the previous *in vivo* observations, where RA showed to repress BBM SGLT1 expression in response to dietary carbohydrates. Despite disclosing the same intracellular location in Caco-2 cells, the expression and/or location of both SGLT1 and GLUT2 were not affected by glucose or insulin. Also in HT-29 cells, insulin did not modify SGLT1 expression suggesting that, the hormone, may not contribute to the translocation of the transporter from intracellular storage sites to the apical membrane.

This work allowed us to conclude that the studied *Salvia* species and their natural compounds may be useful in preventing and controlling diabetes and associated complications, namely dyslipidemia.

## Resumo

A diabetes de mellitus tipo 2 (T2DM) é uma doença metabólica caracterizada por elevados níveis de glucose no sangue (hiperglicemia), como consequência de falhas na secreção e/ou ação da insulina. A T2DM representa um fator de risco para o desenvolvimento de doenças cardiovasculares (DCVs) uma vez que, além da hiperglicemia, os diabéticos apresentam frequentemente níveis anormais de lípidos no sangue (dislipidemia diabética). Com a doença a atingir proporções epidémicas, surge a necessidade de encontrar terapias inovadoras ou complementares às metodologias tradicionalmente usadas no controlo da hiperglicemia e dislipidemia. A presente tese teve como objetivo estudar os efeitos de duas espécies do género de *Salvia* (*S. officinalis* e *S. fruticosa*) na prevenção da diabetes e suas complicações. Este trabalho pretendeu ainda identificar compostos naturais destas plantas medicinais com potencial antidiabético e de redução lipídica e caracterizar os seus mecanismos de ação no fígado e no intestino delgado, importantes alvos na progressão da diabetes.

Com o intuito de estudar o potencial antidiabético do extrato aquoso de *S. officinalis* (preparado como chá), realizou-se um estudo piloto em voluntárias saudáveis que, de acordo com a idade, constituíam um grupo de risco para o desenvolvimento da diabetes (*capítulo 2*). O consumo deste chá melhorou o perfil lipídico e aumentou as defesas antioxidantes das voluntárias, sem no entanto provocar hipoglicemia e/ou hepatotoxicidade. Estas evidências indicam que o chá de *S. officinalis* pode conter compostos benéficos para a prevenção das DCVs, a principal causa de morbilidade e mortalidade em diabéticos.

Num estudo posterior, recorreu-se à manipulação da dieta para avaliar os efeitos dos extratos aquosos de *Salvia fruticosa* (SFT) e do ácido rosmarínico (RA), o principal composto fenólico dos extratos de *S. officinalis* e *S. fruticosa*, em alguns processos do metabolismo lipídico (*capítulo 3*). A dieta pobre em hidratos de carbono (Lc) diminuiu os níveis plasmáticos de colesterol enquanto o SFT, administrado aquando da reintrodução da dieta normal (referida como rica em hidratos de carbono, [Hc]), aumentou os níveis de HDL no plasma. Ambos os tratamentos (SFT e RA) demonstraram efeitos na regulação de fatores de transcrição envolvidos na expressão de

enzimas que controlam a síntese lipídica. Adicionalmente, o RA demonstrou ser eficaz na indução de genes envolvidos na oxidação hepática de ácidos gordos e na prevenção de uma excessiva absorção intestinal de colesterol, como resposta à manipulação da dieta.

O *capítulo 4* é dedicado ao estudo dos efeitos da luteolina-7-glucosideo (L7G), um flavonoide também presente no extrato de *S. officinalis*, no metabolismo lipídico em ratos saudáveis. Os resultados obtidos demonstraram que, tal como o RA (*capítulo* 3), a L7G induz a expressão de genes envolvidos na oxidação de ácidos gordos. Além disso, a L7G diminuiu a expressão da enzima reguladora da síntese endógena de colesterol e sugeriu uma potencial inibição da atividade do SREBP-1 (um fator de transcrição regulador da lipogénese).

Por fim, realizou-se um estudo para avaliar os efeitos da glucose e do RA em células HT-29, diferenciadas com butirato (NaBu) e Caco-2, diferenciadas espontaneamente em cultura. Estas linhas celulares são frequentemente consideradas bons modelos *in vitro* do epitélio intestinal (*capítulo 5*). Verificou-se que o crescimento e o estado de diferenciação de ambas as linhas celulares foram distintamente afetados pela glucose. O isolamento de frações celulares revelou um aumento da expressão proteica do transportador da glucose SGLT1 na membrana apical (BBM) e um enriquecimento do transportador da glucose GLUT2 na fração das restantes membranas intracelulares (incluindo a membrana basolateral, [BLM]) em células HT-29 diferenciadas com NaBu. A expressão do SGLT1 nesta linha celular não foi afetada pelo RA. Esta observação opõe-se aos resultados previamente obtidos *in vivo*, que demonstraram a eficácia do RA para prevenir o aumento dos níveis de SGLT1 na BBM, em consequência da manipulação dos hidratos de carbono na dieta. Os transportadores (SGLT1 e GLUT2) demonstraram possuir a mesma localização intracelular em Caco-2 contudo, a expressão e a localização de ambas as proteínas não foram afetadas pela glucose nem pela insulina. Do mesmo modo, os níveis proteicos e a localização intracelular do SGLT1 em HT-29 não foram afetados pela insulina podendo isto indicar que, esta hormona, não intervém no processo de transporte do SGLT1 intracelular para a membrana apical.

Este trabalho permitiu concluir que as espécies de *Salvia* e os seus compostos naturais presentemente estudados, podem ser eficazes na prevenção e no controlo da diabetes e suas complicações, nomeadamente a dislipidemia.

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# Abbreviations list

| | |
|---|---|
| ACC | Acetyl-CoA carboxylase |
| Akt | Protein kinase B |
| ALP | Alkaline phosphatase |
| ALT | Alanine aminotransferase |
| AMPK | AMP-activated protein kinase |
| Apo | Apolipoprotein |
| AST | Aspartate aminotransferase |
| BBM | Brush-border membrane |
| BLM | Basolateral membrane |
| ChREBP | Carbohydrate responsive element-binding protein |
| CPT | Carnitine palmitoyltransferase (CPT1, CPT2) |
| CVD | Cardiovascular disease |
| ER | Endoplasmic reticulum |
| FAS | Fatty acid synthase |
| FFA | Free fatty acid |
| FPG | Fasting plasma glucose |
| GLUT | Glucose transporter (GLUT2, GLUT4, GLUT5) |
| GRP78/BIP | 78 kDa Glucose-regulated protein/Immunoglobulin-binding protein |
| GSK3 | Glycogen synthase kinase-3 |
| G6Pase | Glucose-6-phosphatase |
| Hc diet | Normal (High carbohydrate) diet |
| HDL | High-density lipoprotein |
| HMGCR | 3-hydroxy-3-methylglutaryl coenzyme A reductase |
| IDL | Intermediate-density lipoprotein |
| IFG | Impaired fasting glycaemia |
| IGT | Impaired glucose tolerance |
| IR | Insulin receptor |
| IRS | Insulin receptor substrate |
| JNK | c-Jun N-terminal kinase |
| Lc diet | Low carbohydrate diet |
| LDL | Low-density lipoprotein |
| LDLR | Low-density lipoprotein receptor |
| LPL | Lipoprotein lipase |
| LRP | Lipoprotein receptor-related protein |

| | |
|---|---|
| LXR | Liver X receptor |
| L7G | Luteolin-7-glucoside |
| MetS | Metabolic syndrome |
| NAFLD | Nonalcoholic fatty liver disease |
| NPC1L1 | Niemann-Pick C1-like 1 |
| OGTT | Oral glucose tolerance test |
| PCNA | Proliferating cell nuclear antigen |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PKC | Protein kinase C (PKC ζ, PKC θ, PKC λ) |
| PPAR | Peroxisome proliferator-activated receptor (PPARα, PPARγ) |
| PI3K | Phosphatidylinositol 3-kinase |
| RA | Rosmarinic acid |
| SFT | *Salvia fruticosa* aqueous extract (infusion, commonly referred as " tea") |
| SGLT1 | Sodium-glucose cotransporter 1 |
| SOT | *Salvia officinalis* aqueous extract (infusion, commonly referred as " tea") |
| SREBP | Sterol regulatory element-binding protein (SREBP-1a, SREBP-1c, SREBP-2) |
| TNF-α | Tumor necrosis factor-alpha |
| T1DM | Type 1 diabetes mellitus |
| T2DM | Type 2 diabetes mellitus |
| UPR | Unfolded protein response |
| VLDL | Very low-density lipoprotein |

*"Eles não sabem que o sonho*
*é uma constante da vida*
*tão concreta e definida*
*como outra coisa qualquer (...)*
*Eles não sabem, nem sonham,*
*que o sonho comanda a vida,*
*que sempre que um homem sonha*
*o mundo pula e avança*
*como bola colorida*
*entre as mãos de uma criança."*

*Pedra Filosofal,*
*António Gedeão*

*"A vida de uma bailarina é cheia de desafios e obstáculos que*
*nos causam dor e sofrimento. Porém não há emoção maior*
*que a glória e o prazer que sentimos no dia de um espectáculo."*

*Michele A. Domingues*

# CHAPTER 1

## *General Introduction*

# 1. Diabetes mellitus

## 1.1. Brief considerations

For long considered as a disease of minor significance, nowadays diabetes mellitus becomes a serious threat to world health and is considered the fourth leading cause of death in developed countries (Nather, 2008; Zimmet, 2000). Diabetes is a metabolic disease characterized by chronic elevated blood glucose levels (hyperglycaemia), caused by the lack in pancreatic insulin production and/or scarce response of the target tissues to insulin (Klover and Mooney, 2004). The number of diabetic patients dramatically increased in the past two decades and is expected to reach 366 million individuals in 2030 (**Figure 1**).

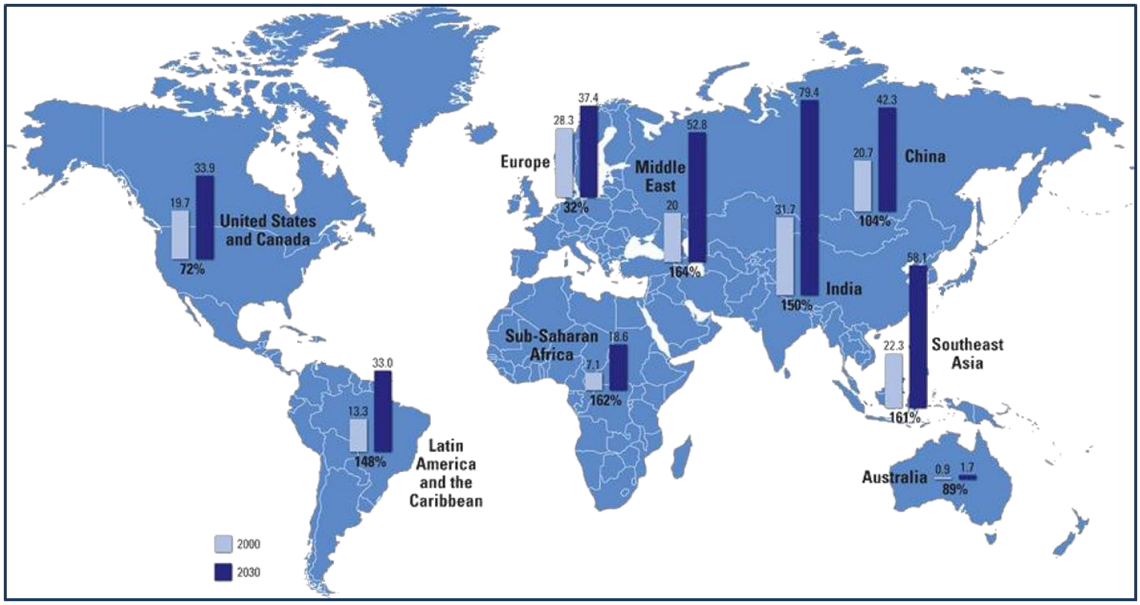


**Figure 1.** Global prevalence of diabetes mellitus with estimated number of people (in million) affected by the disease in 2000 (light blue) and 2030 (dark blue). Adapted from [Hossain et al., 2007; Wild et al., 2004].

Diabetes-associated complications such as diabetic neuropathy, renal failure, cardiovascular diseases (CVDs), amputations and blindness increases disability, reduces life expectancy, and causes enormous health costs and social impact (American Diabetes Association, 2011; Nichols et al., 2000). Therefore, diabetes is one of the most challenging health problems in 21$^{st}$ century (Wild et al., 2004; Zimmet, 2000).

### 1.1.1. Diabetes diagnosis

Diabetes often remains undiagnosed since individuals can experience different warning signs, and most of the times the symptoms seem to be harmless. However, the early detection of diabetes symptoms could prevent the progression of the disease and reduce the change of developing diabetes-related complications. The described symptoms for diabetes include polyuria (excessive production of urine), polydipsia (excessive thirst), blurred vision and polyphagia (extreme hunger), sometimes associated with unexplained weight loss (The expert committee on the diagnosis and classification of diabetes mellitus, 2003).

At least two tests are very common in diabetes diagnosis: the fasting plasma glucose test (FPG) and the oral glucose tolerance test (OGTT) (American Diabetes Association, 2011). The first one evaluates the blood glucose levels in a person in fasting for at least 8 hours. FPG test is the most common used to detect diabetes not only because is cheaper but also due to its convenience. However and despite the less convenience, the OGTT test is more sensitive and reliable than the FPG test for diagnosing diabetes. It allows to clinically diagnosing prediabetic and diabetic people through the evaluation of the efficacy of their body to metabolize glucose. The test requires an overnight fasting (at least 8 hours fasting) and plasma glucose is measured immediately before and 2 hours after the drinking of a standard amount of glucose (usually 75g/300ml of water). The values obtained are further compared. **Table 1** depicts the revised diagnostic reference values for blood glucose levels in fasting and after an OGTT.

**Table 1.** Diagnostic values of diabetes mellitus. Adapted from [Diabetes Educational Services, 2009].

| **Stage** | **FPG** (mmol/l [mg/dl]) | **OGTT** (mmol/l [mg/dl]) |
|---|---|---|
| **Normal** | < 5.6 [< 100] | < 7.8 [< 140], *2h plasma glucose* |
| **Prediabetes** | IFG: ≥ 5.6 and < 7.0 [≥ 100 and < 126] | IGT: ≥ 7.8 and < 11.1 [≥ 140 and < 200] *2h plasma glucose* |
| **Diabetes** | ≥ 7.0 [≥ 126] | ≥ 11.1 [≥ 200], *2h plasma glucose* |

FPG: Fasting plasma glucose; OGTT: oral glucose tolerance test; IFG: impaired fasting glucose; IGT: impaired glucose tolerance.

### 1.1.2. Classification of diabetes mellitus

Diabetes mellitus is classified in different types according to its aetiology and clinical presentation:

- *Type 1 diabetes mellitus (T1DM)*: an idiopathic disease since it usually arises from an autoimmune destruction of the pancreatic β-cells, resulting in little or absolute deficient insulin production (Robertson, 2004; The expert committee on the diagnosis and classification of diabetes mellitus, 2003; Zimmet et al., 2001). Individuals with this type of diabetes became dependent on exogenous insulin for survival in order to control blood glucose levels and avoid ketoacidosis (excessive hepatic ketone bodies production that ultimately leads to diabetic coma) (American Diabetes Association, 2004).

- *Type 2 diabetes mellitus (T2DM)*: a metabolic disease characterized by defects in insulin secretion and/or action, that leads to insulin resistance and consequently to hyperglycaemia (American Diabetes Association, 2011; Cheng and Fantus, 2005; Rydén et al., 2007). T2DM is the most common form of the disease and its prevalence is related with dietary choices, ageing, reduced physical activity and other unhealthy lifestyle patterns (Rydén et al., 2007). Unlike T1DM, people with T2DM are independent on exogenous insulin and, normaly, do not disclose ketoacidosis (American Diabetes Association, 2004; Rydén et al., 2007). Insulin treatment may, however, be required if diet alone or in combination with oral pharmacological agents, fails to control hyperglycaemia (Nyenwe et al., 2001). This type of diabetes could remain asymptomatic for many years leading to its tardily diagnosis, which often occurs casually through abnormal blood and/or urine glucose test or by detecting diabetes-related complications (Conget, 2002). T2DM appears usually after the age 40 although its prevalence has been growing in children and young people (Conget, 2002; Libman and Arslanian, 2003).

- *Gestational diabetes mellitus*: a glucose intolerance with any degree of severity which starts or is first recognized during pregnancy (Conget, 2002; Rydén et al., 2007; The expert committee on the diagnosis and classification of diabetes mellitus, 2003).

- *Other specific types*: include rare forms of diabetes like *Maturity-Onset Diabetes of the Young (MODY)* - caused by mutations in an autosomal dominant gene that leads to ineffective insulin production and release from the pancreas (Conget, 2002); *Diseases of*

*the exocrine pancreas* - caused by pancreatic injuries such as trauma, infection, pancreatitis and pancreatic carcinoma (Hardt et al., 2008) and *Drug or chemical-induced diabetes* – caused by several drugs that affect insulin secretion leading to diabetes onset (Bendz and Aurell, 1999).

### 1.1.3. Diabetes-associated complications

Diabetes is one of the leading causes of morbidity and mortality in developed countries mainly due to its associated complications that include microvascular (diabetic retinopathy, nephropathy and neuropathy) and macrovascular (cardiovascular diseases) complications (American Diabetes Association, 2011; Bloomgarden, 2004; The expert committee on the diagnosis and classification of diabetes mellitus, 2003). The main chronic complications of diabetes are reviewed in **Figure 2**.

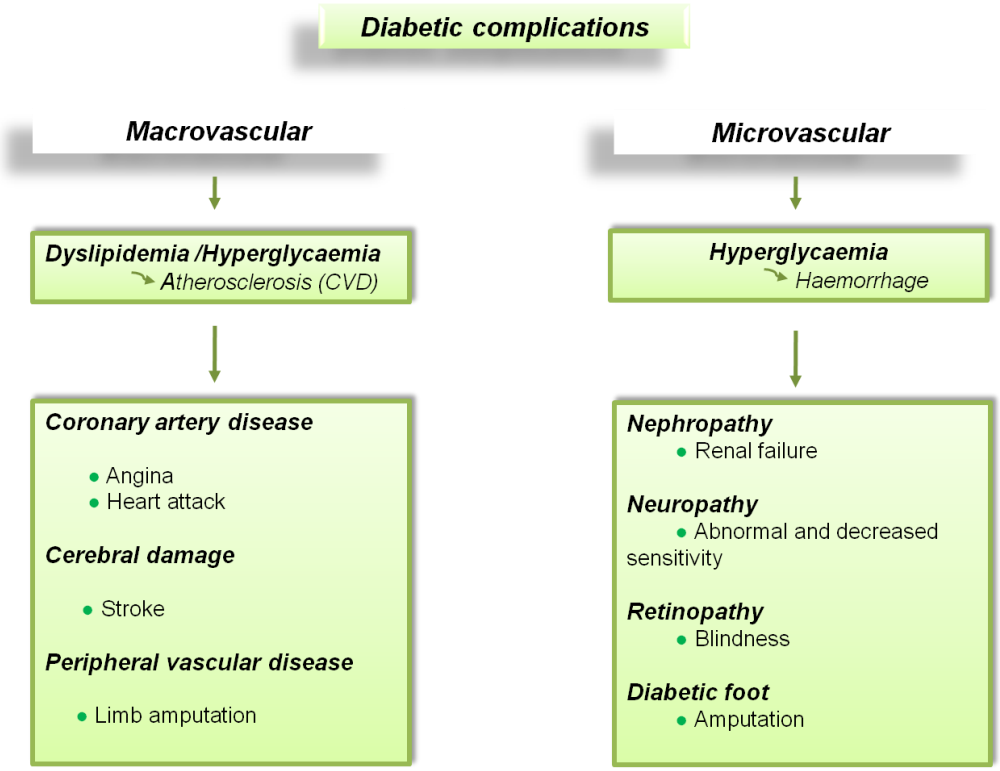


**Figure 2.** Overview of the major diabetes-associated complications and their ultimately consequences.

Patients with diabetes experience significant risk to develop diabetic dyslipidemia, a disturbance characterized by elevated plasma triglyceride and very low-density lipoprotein (VLDL), reduced high-density lipoprotein (HDL), with or without increased low-density lipoprotein (LDL) (Boden and Pearson, 2000; Mooradian, 2009; Smith,

2007). This abnormal lipid profile pattern is strongly associated with increased free fatty acid (FFA) flux due to insulin resistance that prompts undesirable metabolic events within the endothelium, leading to impaired endothelial function, increased vasoconstriction, inflammation and thrombosis stimulation (Beckman et al., 2002; Chahil and Ginsberg, 2006; Mooradian, 2009). During this atherosclerotic process, LDL particles may undergo oxidation and be ingested by monocytes to originate foam cells that, once accumulated within blood vessels, leads to atherosclerotic plaque formation. This may result in ischemia and prompt to CVDs progression (like coronary heart disease and stroke) (Achmad et al., 1997; Jay et al., 2006; Wright et al., 2006).

The hallmark of diabetes, chronic hyperglycaemia, is associated with chronic oxidative stress that harmfully modifies the cellular structure and function of several tissues. This accounts for the development of several diabetic complications that may culminate in blindness, renal failure, limb amputation as well as CVDs onset. Glucose autoxidation, oxidative phosphorylation and protein kinase C (PKC) activation constitute some of the biochemical pathways implicated in this "glucotoxicity" (Robertson, 2004; Robertson and Harmon, 2006).

Since T2DM is considered a preventable disease through lifestyle modification (including healthy diet, physical exercise and weight controlling) in combination with pharmacological interventions, this type of diabetes will be focused in detail in subsequent sections of this dissertation.

## 1.2. Type 2 diabetes mellitus: a multifactorial metabolic disorder

T2DM is a multifactorial, progressive disease clinically manifested by hyperglycaemia as a result of impaired insulin sensitivity of the target tissues (liver, muscle and adipose tissue), a condition called insulin resistance. T2DM is the major responsible for the recent epidemic outbreak of diabetes, accounting for 90 to 95% of the prevalence of diabetes. The associated economic costs are even more eloquent and excluded from those facts is the reduced quality of life not only for diabetic patients, but also to their families and close friends.

The evolution from normal glucose tolerance to impaired glucose tolerance (IGT) and ultimately to T2DM involves genetic and environmental factors (Stumvoll et al., 2005; Surampudi et al., 2009). Although, is not possible to discuss the pathogenesis of

T2DM without considering the role of glucose on this process. Glucose is the primary metabolic source of energy to the body and its homeostasis is balanced between glucose intake (gut absorption), tissue consumption (metabolic processes such as glycolysis, pentose phosphate pathway, tricarboxylic acid cycle and glycogenesis) and intracellular production (gluconeogenesis and glycogenolysis) (Meyer et al., 2002).

In order to avoid chronic complications, blood glucose levels are maintained within a narrow range by the body's homeostatic mechanisms: a hormonal system in which insulin and glucagon are the protagonists (Desvergne et al., 2006; Kawahito et al., 2009). Insulin is produced and secreted by the pancreatic β-cells (**Figure 3**) in response to increased circulating glucose and amino acid levels after feeding (Sesti, 2006). Once released, this anabolic hormone is crucial for glucose homeostasis since it induces glucose uptake (chiefly in skeletal muscle and adipose tissue) and suppresses endogenous hepatic glucose production, through decreasing both gluconeogenesis (glucose production) and glycogenolysis (glycogen degradation). Furthermore, insulin is also involved in lipid metabolism since it inhibits lipolysis (triglyceride degradation) and consequent fatty acid discharge from adipose tissue, favouring lipid synthesis (lipogenesis) in liver and fat cells (DeFronzo, 2004; Sesti, 2006).

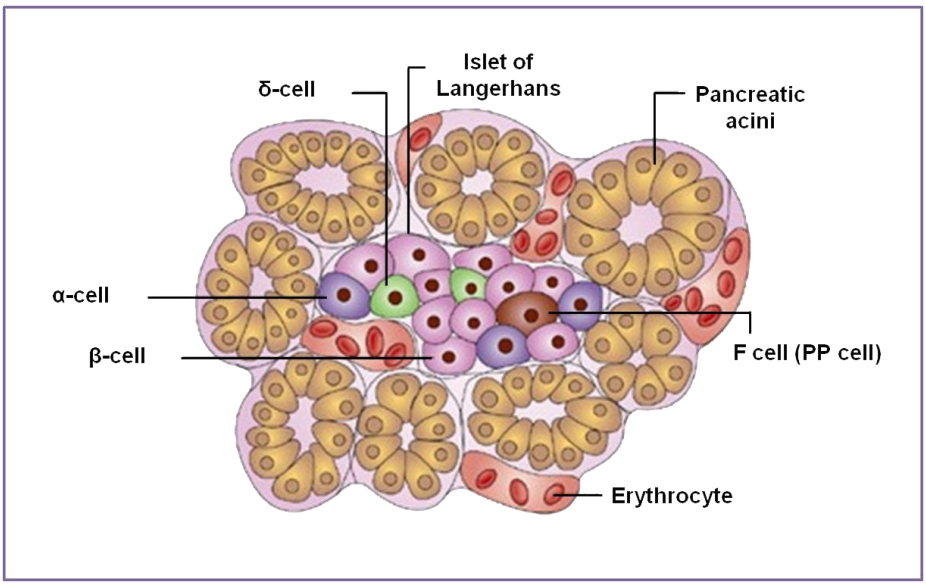


**Figure 3.** Schematic representation of the pancreatic cells. Adapted from [Bardeesy and DePinho, 2002].

Glucagon is a counter-regulatory hormone that antagonizes insulin's action mainly in the liver. In postabsorptive state (overnight fasting) or between meals, the

decrease of blood glucose to levels below the physiological range (lower than 4mM) leads to glucagon production and secretion by the pancreatic α-cells (**Figure 3**). Once released, this catabolic hormone induces the hepatic output and discharge of glucose by increasing glycogenolysis (during the first 8-12 hours of fasting) and gluconeogenesis (over longer periods of fasting) (Aronoff et al., 2004). The increase of blood glucose levels after a meal leads to the inhibition of glucagon production/release due to the increase of blood insulin levels (see section 1.4.1).

### 1.2.1. Insulin signalling pathways

Insulin is considered the key hormone for controlling critical energy functions such as glucose and lipid metabolism. At the cellular level, insulin affects vesicle trafficking, induces protein phosphatases and kinases, regulates gene expression and promotes cell growth and differentiation (Saltiel and Pessin, 2002). This intricacy indicates that insulin operates in multiple signalling pathways through the activation of its tyrosine kinase receptor (Saltiel and Pessin, 2002).

Insulin signalling engages a cascade of molecular events initiated by the binding of this hormone to the extracellular α-subunit of its cell surface receptor, the insulin receptor (IR) (**Figure 4**). This induces a conformational change that directs to the autophosphorylation of numerous intrinsic tyrosine residues present in transmembrane β-subunit of the IR (Asante-Appiah and Kennedy, 2003; Choi and Kim, 2010; Sesti, 2006). These residues are then recognised by different substrate adaptor proteins including the members of the insulin receptor substrate family (IRS) (Pessin and Saltiel, 2000; Saltiel and Kahn, 2001). This recruitment and consequent phosphorylation results in IRS binding and activation of numerous signalling partners among them, the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K). PI3K is a heterodimeric lipid kinase that has become a major focus of attention due to its central role in cancer progression and insulin's metabolic responses (especially the PI3K class Ia) (Bertrand et al., 2008; Zhao and Vogt, 2008). Once activated, the catalytic p110 subunit of PI3K catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3) which stimulates phosphoinositide-dependent kinase-1 (PDK1) activity (**Figure 4**). This conversion of PIP2 into PIP3 could be reverted by the negative regulator phosphatase and tensin homologue deleted

on chromosome 10 (PTEN). A key downstream effector of PDK1 is Akt (the well-known protein kinase B), but PDK1 is also capable of activating the atypical PKC isoforms ζ and λ (PKC ζ/λ) (Biddinger and Kahn, 2006; Montecucco et al., 2008; Sesti, 2006).

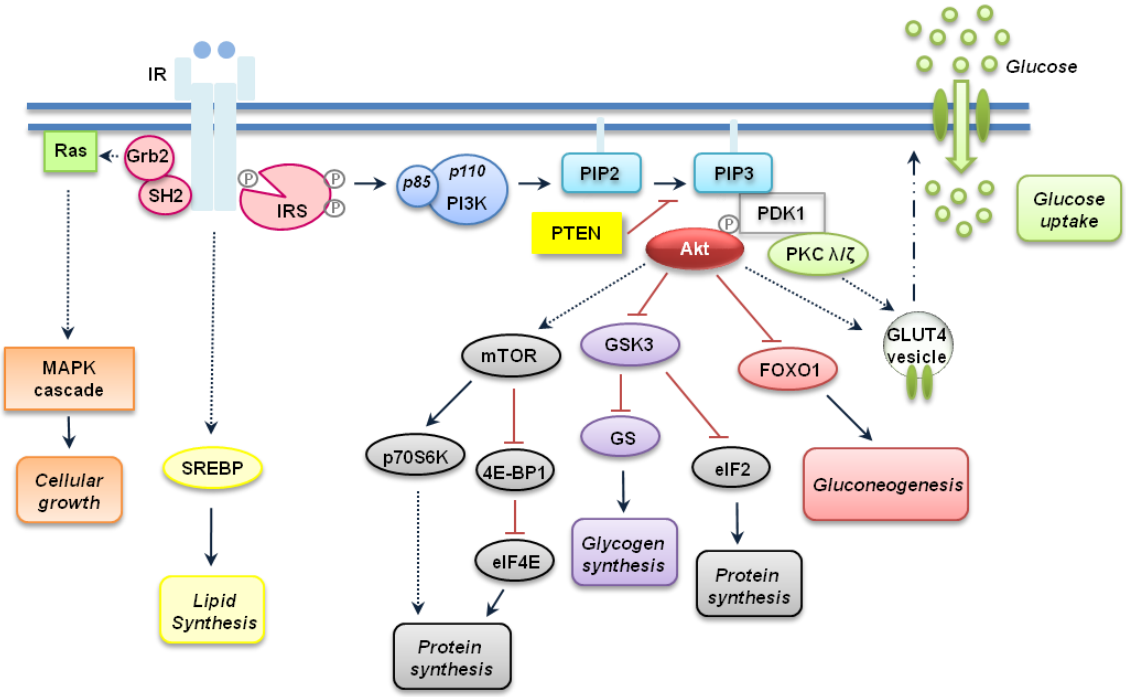


**Figure 4.** Schematic representation of insulin signalling pathway. IR: insulin receptor; IRS: insulin receptor substrate; PI3K: phosphatidylinositol 3-kinase (subunity p85 and subunity p110); PIP2: phosphatidylinositol 4,5-bisphosphate; PIP3: phosphatidylinositol 3,4,5-trisphosphate; PDK1: phosphoinositide-dependent kinase-1; PTEN: phosphatase and tensin homologue deleted on chromosome 10; Akt: protein kinase B; PKC ζ/λ: atypical protein kinase C; GLUT4: glucose transporter 4; FOXO1: forkhead box O1; GSK3: glycogen synthase kinase-3; GS: glycogen synthase; eIF2B: eukaryotic initiation factor 2B; mTOR: mammalian target of rapamycin; p70S6K: p70 ribosomal S6 kinase; 4E-BP1: eukaryotic translation initiation factor 4E binding protein 1; eIF4E: eukaryotic initiation factor 4E; SREBP: sterol regulatory element-binding protein; SH2: Src homology 2 domain; Grb2: growth factor receptor binding protein-2; Ras: GTP-binding protein; MAPK: mitogen-activated protein kinase; Ⓟ: phosphorylation.

In fat and muscle cells, activation of Akt induces the phosphorylation of its substrate (AS160) that leads to glucose uptake into cells by promoting glucose transporter 4 (GLUT4) mobilization from intracellular stores to the cell surface (**Figure 4**). GLUT4 translocation is also regulated by the atypical PKC ζ/λ isoforms (Choi and Kim, 2010). A PI3K-independet pathway has been proposed to provide a second signal for regulating GLUT4 trafficking into cell surface athough, the importance of this pathway in insulin-stimulated glucose uptake remains controversial (Chang et al., 2004; Mitra et al., 2004; Watson and Pessin, 2007; Zhou et al., 2004).

Activated Akt also induces a cascade of events that culminates in glycogen synthesis (glycogenesis) through inactivation of glycogen synthase kinase-3 (GSK3), protein synthesis via the mammalian target of rapamycin (mTOR) and cell survival throughout the inhibition of several proapoptotic molecules. Under basal conditions, the constitutive activity of GSK3 results in inhibitory phosphorylation of glycogen synthase (GS) protein, the enzyme responsible for promoting glucose storage as glycogen. Upon insulin stimulation, phosphorylated Akt induces inhibitory phosphorylation of the regulatory Serine 21 or 9 residue of GSK3 (α and β, respectively), activating GS and consequently, glycogen synthesis (Lee and Kim, 2007) (**Figure 4**).

Protein synthesis is promoted by insulin-mediated Akt activation through two distinct mechanisms (**Figure 4**). One mechanism engages the inhibitory phosphorylation of GSK3 and consequent dephosphorylation of eukaryotic initiation factor 2B (eIF2B) (Welsh et al., 1997). This guanine nucleotide exchange factor induces protein synthesis from amino acids since it regulates the initiation steps of protein translation (Lee and Kim, 2007; Lizcano and Alessi, 2002). The other mechanism involves Akt-mediated direct phosphorylation and activation of mTOR that leads to p70 ribosomal S6 kinase (p70SK6) activation (Saltiel and Kahn, 2001) and inhibition of the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) (Asnaghi et al., 2004). Once inactivated, the translation inhibitor 4E-BP1 promotes the activation of the eukaryotic initiation factor 4E (eIF4E) and translation occurs (Asnaghi et al., 2004; Saltiel and Kahn, 2001).

In addition to promoting glucose storage, insulin suppresses the hepatic production and secretion of glucose via blocking gluconeogenesis and glycogenolysis (Saltiel and Kahn, 2001). The phosphorylation of the transcription factor forkhead box O1 (FOXO1) by Akt, suppresses the transcription of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), key enzymes involved in the gluconeogenic process (Biddinger and Kahn, 2006; Leclercq et al., 2007).

Insulin also plays a role in regulating lipid metabolism by promoting lipogenesis instead of lipolysis (**Figure 4**). The mechanism behind this regulation seems to be dependent of the activation of the sterol regulatory element-binding protein-1c (SREBP-1c) (Desvergne et al., 2006). This transcription factor regulates the expression of several lipogenic enzymes involved in fatty acid synthesis such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) (Azzout-Marniche et al., 2000; Foretz et al., 1999a;

Saltiel and Kahn, 2001). It has also been reported that insulin-stimulated SREBP-1c expression inhibits the hepatic transcription of PEPCK, suggesting a role of SREBP-1c on the regulation of glucose metabolism by controlling gluconeogesis (Chakravarty et al., 2004; Yamamoto et al., 2004). Repression of the hepatic ketogenesis (a process by which fatty acids are converted into ketone bodies to serve as a fuel for the brain during prolonged starvation), stimulation of hepatic triglycerides synthesis and storage in adipose tissue are additional insulin functions (Karam, 1997).

Finally, activation of the IR also drives the activation of mitogen-activated protein kinase (MAPK) pathway (**Figure 4**). Controlled regulation of this cascade of phosphorylation events is critical for cell proliferation and differentiation. However, unregulated activation can result in oncogenesis.

### 1.2.2. Insulin resistance

Any derangement in insulin signalling can eventually lead to inhibition of its effects and guide to insulin resistance. As afore mentioned, insulin resistance is a condition in which the sensitivity of the target cells to normal circulating levels of insulin is decreased and it is a hallmark of T2DM (Montecucco et al., 2008). This state is known to be present in insulin-sensitive tissues such as skeletal muscle, liver, kidney and adipose tissue, previous to the onset of hyperglycaemia. When these tissues fail to effectively respond to normal circulating insulin levels, blood glucose levels raise leading to hyperglycaemia. Beta-cells continue to produce and secrete insulin as an attempt to improve insulin response of peripheral tissues (Guillausseau et al., 2008; Robertson and Harmon, 2006). Nevertheless, this compensatory mechanism fails and hyperglycaemia aggravates, leading to β-cell exhaustion and eventually apoptosis, culminating in impaired insulin production and secretion. As hyperglycaemia aggravates, prolonged exposure to elevated glucose levels becomes deleterious to normal cell metabolism and function (Robertson, 2004; Robertson and Harmon, 2006).

Defects in IR including gene expression, protein activity or even in phosphorylation status have been implicated in insulin resistance (Pessin and Saltiel, 2000). Alterations on the phosphorylation status of IR could influence its expression, ligand binding and tyrosin kinase activity and impair insulin signalling (Pessin and Saltiel, 2000). Several other events, such as reduced PI3K activity and induced atypical PKC activation, are negative regulators of the insulin signalling (Kruszynska et al.,

2002; Montecucco et al., 2008; Sesti, 2006). The role of Akt in insulin resistance remains, however, controversial. While some studies reported that skeletal muscle from T2DM patients and obese subjects have decreased insulin-induced Akt activation (Beeson et al., 2003; Brozinick et al., 2003; Krook et al., 1998), others did not succeed to detect changes in Akt activation (Beeson et al., 2003; Kim et al., 1999; Krook et al., 2000). Defects on GLUT4 gene expression seems not to be critical for impaired insulin sensitivity. Although, alterations on its translocation machinery including recruitment, docking and fusion of GLUT4-containing vesicles to the plasma membrane of muscle and adipose tissue, seems to play a role on insulin resistance (Montecucco et al., 2008; Zierath et al., 2000).

Nonetheless, insulin resistance is a complex condition that involves intermediates other than those drawn in the insulin pathway, in which the molecular mechanisms underlying this state are not completely understood (Montecucco et al., 2008). Inflammatory molecules (like interleukin 1 and tumor necrosis factor-alpha [TNF-α]) are believed to play a role in the pathogenesis of insulin resistance, by increasing blood glucose levels, impairing β-cell secretion or affecting IRS activity (Herder et al., 2009; Larsen et al., 2007; White, 2002).

Given the pivotal role of lipid metabolism in the pathogenesis of insulin resistance, the alteration of the expression “diabetes mellitus” to “diabetes lipidus” or “diabetes lipomellitus” has been proposed (Shafrir and Raz, 2003). It is widely accepted that increased fatty acids or its metabolites play a critical role in many insulin resistant states such as obesity and T2DM (Saltiel and Kahn, 2001; Wong and Sul, 2010). Elevated blood FFA levels are associated with hepatic and muscular accumulation of triglycerides and fatty acid metabolites, such as diacylglycerol and ceramides (Saltiel and Kahn, 2001). FFA stimulates serine phosphorylation of IRS proteins, which results in impaired insulin sensitivity (Gual et al., 2003; Yu et al., 2002a). In addition, FFA stimulates gluconeogenesis by supplying energy or inducing key gluconeogenic enzymes, leading to increased levels of insulin (hyperinsulinemia) and enhanced triglycerides production (Staehr et al., 2003). Diacylglycerol accumulation and consequent activation of PKC isoenzymes (especially PKC θ) induces β-cell dysfunction, leading to impaired insulin sensitivity and ultimately to insulin resistance (Shafrir and Raz, 2003). PKC θ is also associated with several pathogenic mechanisms involved in the development of atherosclerosis such as endothelial dysfunction and

monocytes-mediated uptake of oxidized LDL particles (Rask-Madsen and King, 2005). Finally, FFA has also been positively associated with nuclear factor κB (NFκB) and c-Jun N-terminal kinase (JNK), which negatively regulates insulin pathway (DeFronzo, 2010; Itani et al., 2002).

Therefore the complexity of insulin resistance is such that glucose and lipid metabolism but also inflammatory events are known to contribute to its onset. In addition to T2DM, insulin resistance plays a major role in the pathogenesis of several other disorders including the metabolic syndrome (MetS), the nonalcoholic fatty liver disease (NAFLD) and is also a feature of the endoplasmic reticulum stress.

#### 1.2.2.1. Insulin resistance and the metabolic syndrome

The metabolic syndrome (MetS, also known as syndrome X), is a cluster of cardiometabolic risk factors that comprises several disturbances such as abdominal obesity (Després, 1998; Lemieux, 2001), hyperglycaemia (Balkau et al., 2007), dyslipidemia (Deen, 2004), hypertension (Marre et al., 2001) and inflammation (Ahmad I. and Miller M., 2001) (**Figure 5**). Several factors have been indicated as roots of the MetS but insulin resistance and consequently hyperinsulinemia, are considered direct causes of other MetS risk factors (Grundy et al., 2004; Saely et al., 2005). In addition to this, the global obesity epidemic has been considered the most important driving force behind the increased incidence of MetS (Ford et al., 2004). MetS is also considered a stronger predictor for diabetes and CVDs (Borgman and McErlean, 2006; Isomaa et al., 2001; Lorenzo et al., 2003).

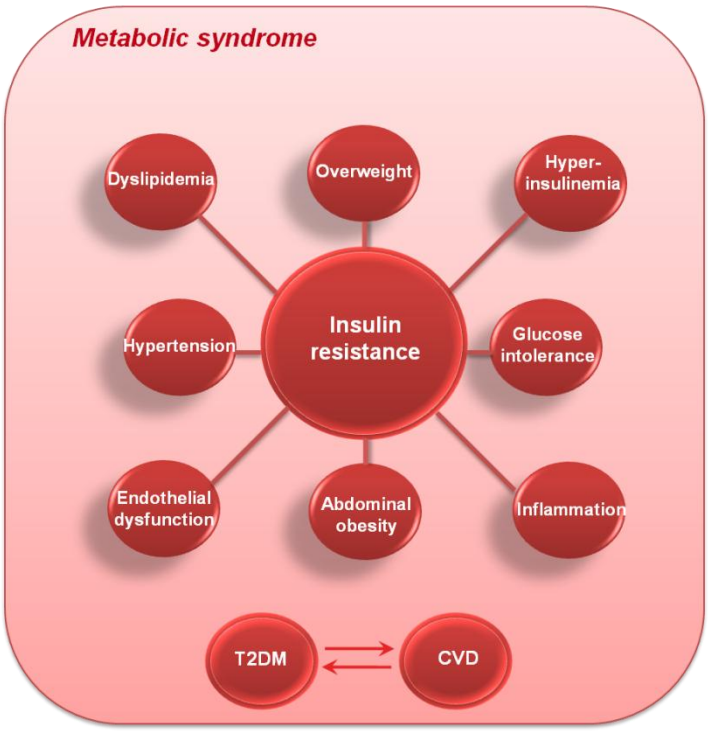


**Figure 5.** The cluster of conditions that characterizes the metabolic syndrome.

Multiple potential mechanisms of insulin resistance have been proposed for MetS onset, including: 1) decreased production of nitric oxide (a potent vasodilator), which leads to endothelial dysfunction (Wang et al., 2004); 2) increased lipolysis in adipocytes that results in increased FFA release and influx to other tissues, like the liver that becomes fatty (Eckel et al., 2005); 3) increased VLDL production and triglyceride levels, leading to a raise in blood pressure (Grundy, 2004) and 4) activation of the MAPK signalling pathway, which results in increased activity of growth promoting agents, a potential source of the proatherogenic mechanism (Wang et al., 2004).

The prevalence of MetS is increasing as a result of increased obesity and ageing (Chew et al., 2006). Management of MetS should initially include lifestyle modifications such as increased physical activity, healthy diet and weight loss, in order to prevent CVDs and diabetes. Physical exercise ameliorates insulin resistance, improves dyslipidemia and decreases visceral obesity, contributing to decrease the incidence of diabetes and MetS (Braith and Stewart, 2006; Knowler et al., 2002). However, pharmacological interventions should be considered to treat MetS more aggressively. Since it has not a single pathogenic mechanism, there is not a unique and specific treatment for this syndrome. The current pharmacological treatments for MetS includes LDL cholesterol-lowering agents (like statins), cholesterol absorption inhibitors (such as ezetimibe), and insulin sensitizing agents (like metformin and thiazolidinediones) (Gerstein et al., 2006; Knowler et al., 2005; Orchard et al., 2005; Tota-Maharaj et al., 2010). Their mechanisms of action and will be discussed in further sections of this thesis.

#### 1.2.2.2. Insulin resistance and nonalcoholic fatty liver disease

Considered the hepatic expression of the MetS, NAFLD is strongly associated with insulin resistance (in liver and adipose tissue) as well as with T2DM and obesity (Bugianesi et al., 2005; Marchesini et al., 2001; Seppälä-Lindroos et al., 2002). NAFLD is a chronic and common hepatic disease characterized by fat deposition in the liver, in the absence of excessive alcohol ingestion and others known causes of fat accumulation such as viral and autoimmune hepatitis (Ali and Cusi, 2009; Utzschneider and Kahn, 2006; Vanni et al., 2010). NAFLD is also a spectrum of diseases ranging from steatosis,

to nonalcoholic steatohepatitis and cirrhosis that ultimately may lead to hepatocarcinoma (Lewis and Mohanty, 2010).

The pathogenesis and progression of NAFLD is explained based on a "multi-hit" hypothesis, since it is a complex process that comprises several steps (Jou et al., 2008; Lewis and Mohanty, 2010). Insulin resistance is responsible for initiating the first "hit" by inducing alterations in lipid metabolism including enhanced lipolysis in peripheral tissues, increased hepatic FFA uptake and trygliceride synthesis. This fat accumulation results in hepatic steatosis (Day and Saksena, 2002; Utzschneider and Kahn, 2006; Vanni et al., 2010). Furthermore, multiple "second hits" including oxidative stress, inflammatory molecules (TNF-α and adipocytokines) and atypical hepatic apoptosis, results in chronic inflammation or cirrhosis (Lewis and Mohanty, 2010; York et al., 2009).

Currently and contrarily to other hepatic disorders, there is no specific therapy for NAFLD. The management of this disease spectrum is limited to approaches that reduce risk factors (obesity, dyslipidemia and insulin resistance) such as lifestyle and pharmacological interventions (metformin, antioxidants, thiazolidinediones, statins and fibrates) (Adams and Angulo, 2006; Orchard et al., 2005; Torres and Harrison, 2008).

#### 1.2.2.3. Insulin resistance and endoplasmic reticulm stress

The endoplasmic reticulum (ER) is a dynamic organelle that plays a critical function in protein folding and secretion, lipid synthesis, and calcium ($Ca^{2+}$) storage and secretion (Anelli and Sitia, 2008; Ni and Lee, 2007). The ER responds to changes in cellular homeostasis; any deviation threatens cell survival and induces ER stress (Eizirik et al., 2008). Multiple cellular disturbances such as disrumptions of cellular redox, $Ca^{2+}$ depletion, protein mutations and viral infection, leads to the accumulation of unfolded or misfolded proteins that aggregate in the ER lumen (Bernales et al., 2006; Eizirik et al., 2008). This imbalance between the ER protein load and folding capacity triggers the activation of a signal response, the unfolded protein response (UPR) (Eizirik et al., 2008; Marciniak and Ron, 2006).

The UPR response comprises three ER stress transmembrane transducers: the inositol-requiring enzyme 1 (IRE1), the PKR-like ER kinase (PERK) and the activating transcription factor 6 (ATF6) (Bernales et al., 2006; Schröder and Kaufman, 2005). Under ER homeostasis, these three sensors are maintained inactivated through their

binding to the ER chaperone 78 kDa glucose-regulated protein/immunoglobulin-binding protein (GRP78/BIP) (Rutkowski and Kaufman, 2004; Wu and Kaufman, 2006). The accumulation of unfolded and/or misfolded proteins in the ER lumen leads to GRP78/BIP recruitment, which dissociates and activates the UPR sensors. Once activated, these sensors act to restore ER homeostasis. Therefore, the primary function of the UPR is to adapt the ER to the changing environment and lessen ER stress, by re-establishing its normal function and prevent cell death. This is achieved by some coordinated main responses: 1) prevention of protein synthesis and translocation into the ER, which decreases protein load that enters into this organelle; 2) increase the transcription of the UPR target genes, such as ER chaperones that increase the folding capacity of ER and 3) increase the proteasomal degradation of misfolded proteins (Ron and Walter, 2007). If, however, these responses fail to restore ER homeostasis, apoptotic cell death is triggered (Bernales et al., 2006; Ron and Walter, 2007). Thus, the UPR system is activated to ensure that normal tissue functions are maintained however, chronic ER stress seems to sense for disease progression such as diabetes and NAFLD (Thomas et al., 2010).

Insulin resistance plays an important role in the ER stress-associated diabetes development. ER stress may activate JNK that phosphorylates the serine residues of the IRS, while repressing tyrosin phosphorylation of this protein, and impairs insulin signalling pathway (Hetz et al., 2011; Weickert and Pfeiffer, 2006). Recent evidences suggested also a link between the ER stress and the NAFLD progression by three major mechanisms: 1) through direct activation of SREBP-1c that induces lipogenesis (Kammoun et al., 2009a; Kammoun et al., 2009b); 2) through repressing triglyceride secretion by the liver (Ota et al., 2008) and 3) through enhancing lipolysis in adipose tissue that accounts for the hepatic triglyceride storage. Moreover, overexpression of GRP78/BIP chaperone improved hepatic steatosis and insulin sensitivity in ob/ob mice, by repressing ER stress-associated SREBP-1c induction and lipogenesis (Kammoun et al., 2009a).

Taking into consideration the critical role of the small intestine and the liver on glucose and lipid homeostasis, the following sections of this dissertation will introduce some of their functional and regulatory features, highlighting the importance of consider both organs as emerging therapeutical targets.

## 1.3. The small intestine: some morphological and functional aspects

The small intestine is the longest portion of the gastrointestinal tract and is constituted by three segments that connect the pylorus to the colon: the duodenum, jejunum and ileum. The small intestine plays an important role on the final steps of enzymatic digestion of fats, proteins and carbohydrates, but also on the absorption of nutrients, resulting from this process, as well as water and electrolytes, from the intestinal lumen into the bloodstream and lymph (Caspary, 1992). The absorption process and a large part of the enzymatic digestion take place at the small intestinal epithelial cells, and requires an increased absorptive surface area (Despopoulos and Silbernagl, 2003). This requirement is achieved due to three important modifications on mucosa structure of the small intestine:

*Kerckring folds* – (also called plicae circulars) are circular mucosal folds that slow food passage and increase surface area for absorption;

*Villi* – (singular, villus) are large fingerlike protrusions of the intestinal wall. At the base of villi is possible to observe deep cavities, called crypts of Lieberkühn, where epithelial cells are generated;

*Microvilli* – microscopic fingerlike protusions of the epithelial cells that, in turn, cover each villus.

The small intestinal epithelium is renewed constantly since many cellular processes like proliferation, differentiation, maturation and apoptosis occur along the crypt-villus axis (Shirazi-Beechey et al., 2010; Yen and Wright, 2006). As they migrate and differentiate towards the villus tip, epithelial cells are extruded into the lumen in a process that takes about 2-5 days after rising out from the crypt (Ferraris, 2001).

In intestinal crypts the proliferative, undifferentiated stem cells originate the differentiated cells that characterize a mature small intestinal epithelium: goblet cells, enteroendocrine cells, Paneth cells and absorptive cells (Clatworthy and Subramanian, 2001) (**Figure 6**). Goblet cells, located along the epithelium, are responsible for secreting mucus that protects and lubrificates the epithelial layer. Paneth cells produce important molecules that maintain the gastrointestinal barrier such as growth factors, digestive enzymes and antimicrobial substances. Contrarily to the others, Paneth cells complete their differentiation at the crypt base, being phagocytosed afterwards (about 23 days later) (Cheng and Leblond, 1974; Snoeck et al., 2005). The enteroendocrine cells represent about 1% of all epithelial cells and are responsible for secreting several

hormones such as glucagon-like peptide 2 and the incretins glucagon-like peptide-1 and glucose-dependent insulinotropic peptide (Shirazi-Beechey et al., 2010).

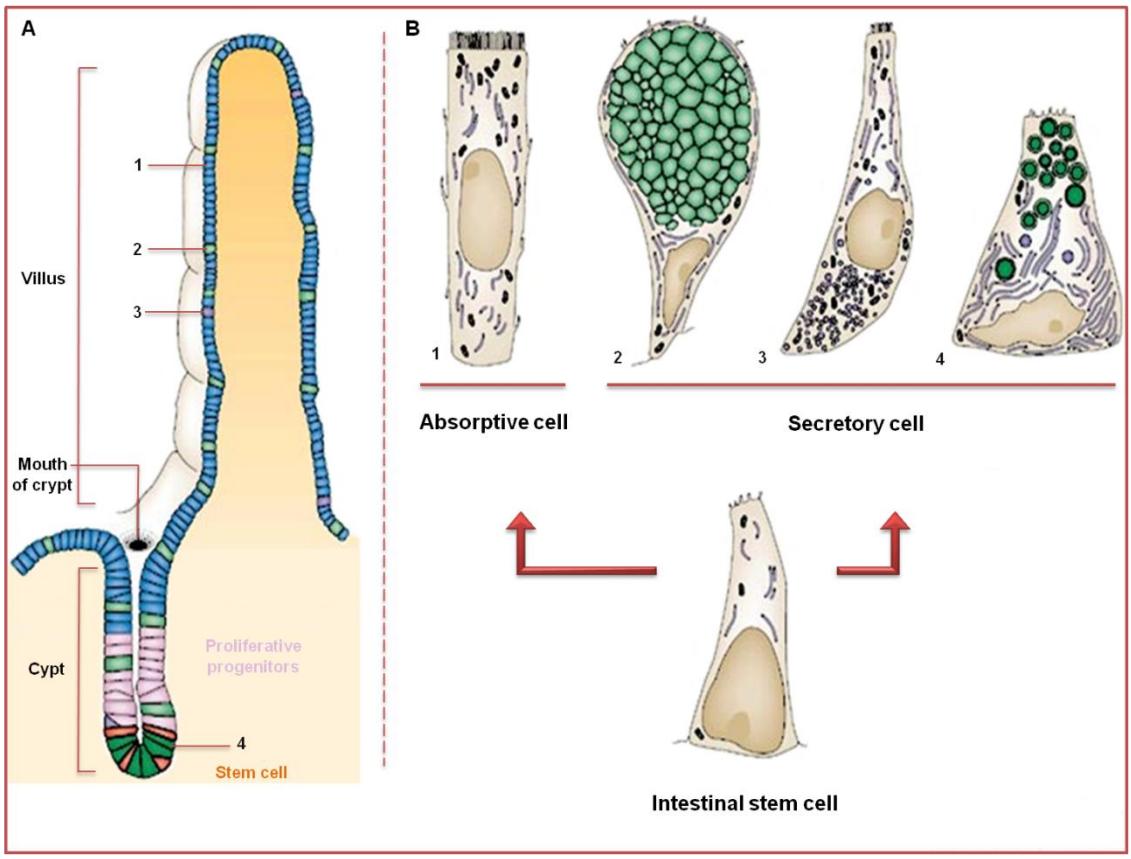


**Figure 6.** Schematic representation of a crypt-villus region (A) and epithelial cell types (B) in the small intestine. 1- Absorptive cell (enterocyte); 2- Goblet cell; 3- Enteroendocrine cell; 4- Paneth cell. Adapted from [Crosnier et al., 2006].

The absorptive cells, the well-known enterocytes, are the most abundant cells in villus, representing about 90% of all epithelial cells (Dauça et al., 1990). These highly polarized cells have an apical membrane (brush-border membrane, [BBM]) and a basolateral membrane (BLM) domain that allows an efficient transport of nutrients from intestinal lumen to the blood (Ferraris, 2001; Shirazi-Beechey et al., 2010). Enterocytes are linked together by tight junctions that maintain the polarity of this epithelial linage and helps to control nutrient, electrolyte and water absorption (Fries et al., 2008; Turner, 2006).

### 1.3.1. Intestinal glucose transporters

Enterocytes differentiate as they migrate toward the villus tip and express carbohydrate digestive enzymes and sugar transport proteins (Cheeseman, 2010). Dietary carbohydrates are digested into simplest forms (monosaccharides) in the small intestine by the intestinal BBM disaccharidases (lactase, maltase and sucrase) and also by pancreatic enzymes (α-amylase) (Dyer et al., 2002). The resultant monosaccharides are absorbed across the apical membrane of enterocytes through sodium ($Na^+$)-dependent (SGLT family) or independent (GLUT family) membrane protein transporters.

The SGLTs comprise a large family of membrane proteins that transports glucose, amino acids, vitamins and electrolytes across the intestinal and renal BBM (Neumiller et al., 2010). The $Na^+$/glucose cotransport hypothesis was postulated for the first time by Crane and its colleagues (1961). The authors defended that glucose is actively transported across the intestinal ephethelium in process that requires a $Na^+$ gradient (Crane et al., 1961). The piooner work done by Crane was followed by many studies that allowed to characterize and understand the $Na^+$ -coupled cotransport.

The first $Na^+$/glucose cotransporter isoform to be cloned was the rabbit SGLT1 followed by the human analogue a few years later (Hediger et al., 1987; Hediger et al., 1989; Wright et al., 2007). The secondary structure of SGLT1 encloses 14 transmembrane α-helices with N and C terminus (facing the extracellular side of the cellular membrane), two phosphorylation sites and a single glycosilation site (Drozdowski and Thomson, 2006; Panayotova-Heiermann et al., 1997; Turk and Wright, 1997). SGLT1 is predominantly expressed in the BBM of the enterocytes and is responsible for transporting glucose and galactose through these epithelial cells.

Like many proteins, SGLT1 is submitted to posttranslational processes that modulate its expression, activity or even function. The posttranslational events comprise glycosylation at the ER, transport to the Golgi apparatus, sorting into the appropriated domains in the plasma membrane, protein turnover and recycling. Some authors have reported that the intracellular RS1 protein (RSC1A1 gene) regulates SGLT1 trafficking, incorporation and retrieval into plasma membrane (Reinhardt et al., 1999; Valentin et al., 2000). Moreover, removing RS1 in mice results in obesity and overexpression of the intestinal SGLT1 (Osswald et al., 2005). In addition, the serum and glucocorticoid inducible kinase isoforms 1 and 3 (SGK1 and SGK3) also modulates intestinal glucose

absorption mediated by SGLT1 (Lang et al., 2006; Nasir et al., 2010; Sandu et al., 2005). In fact, increased SGK1 activity has been associated with enhanced SGLT1 activity, T2DM and MetS development (Dieter et al., 2004; Schwab et al., 2008). In 2010, Nasir and collaborators found that the dietary fibre Gum Arabic (from *Acacia Senegal*) inhibit the intestinal glucose absorption stimulate. However, this effect was not due to a direct interference with the SGLT1 carrier but to a stimulation of RS1 and decrease of SGK3 expression, which regulate SGLT1-vesicle trafficking and protein stability (Nasir et al., 2010).

GLUT family comprises several protein transporters that utilize the diffusion gradient of sugares across plasma membranes of target tissues (Amidon et al., 2002; Brown, 2000). As the other members of the family, GLUT2 has 12 transmembrane domains containing intracellular N and C terminals but of all, GLUT2 is the one that possess the highest Km value for glucose (> 50 mM), explaining its high transport capacity for that sugar (Amidon et al., 2002; Drozdowski and Thomson, 2006). Besides glucose, GLUT2 has also the ability to transport fructose (Barone et al., 2009; Sakar et al., 2009) and is predominantly expressed in liver, pancreatic β-cells, intestinal absorptive cells and kidney proximal tubule (Bouché et al., 2004; Brown, 2000). GLUT2 is found at the intestinal BLM and contributes for the enterocytic transcellular transport of glucose and fructose (Wood and Trayhurn, 2003).

Firstly cloned by Burant and colleagues (1992), GLUT5 is a specific transporter for fructose localized not only in the intestine and testis, but also in kidney, skeletal muscle, adipose tissue and brain (Burant et al., 1992; Hajduch et al., 1998; Hajduch et al., 2003; Mate et al., 2001; Sasaki et al., 2004). Like SGLT1, GLUT5 is expressed at the BBM of intestinal epithelium where it mediates the uptake of fructose, but not of glucose or galactose as the former (Burant and Saxena, 1994; Douard and Ferraris, 2008).

#### 1.3.1.1. Intestinal sugar uptake

Wright and its colleagues (2003) have proposed a simplified six-state kinetic model for the $Na^+$/glucose cotransport (symport) by SGLT1 (**Figure 7**). The process

begins with the binding of two $Na^+$ ions to SGLT1 at the luminal side of the BBM. This induces a conformational change that allows glucose to bind (Wright et al., 2007; Wright et al., 2003). Two $Na^+$ ions and one glucose molecule enter the enterocyte and dissociate from SGLT1 owing to the low intracellular concentration of $Na^+$. The cycle is completed with the reorientation of the empty binding sites of the carrier to the external surface (Drozdowski and Thomson, 2006; Wright et al., 2007). This process yields one mole of glucose and two moles of cations ($Na^+$) that across the enterocyte, and is accompanied by water and two moles of anions that ensure the electroneutrality of the process. The required energy gradiente for the whole process is maintained by the $Na^+/K^+$-ATPase, localized at the BLM of the enterocytes (Drozdowski and Thomson, 2006; Wright et al., 2007; Wright et al., 2003).

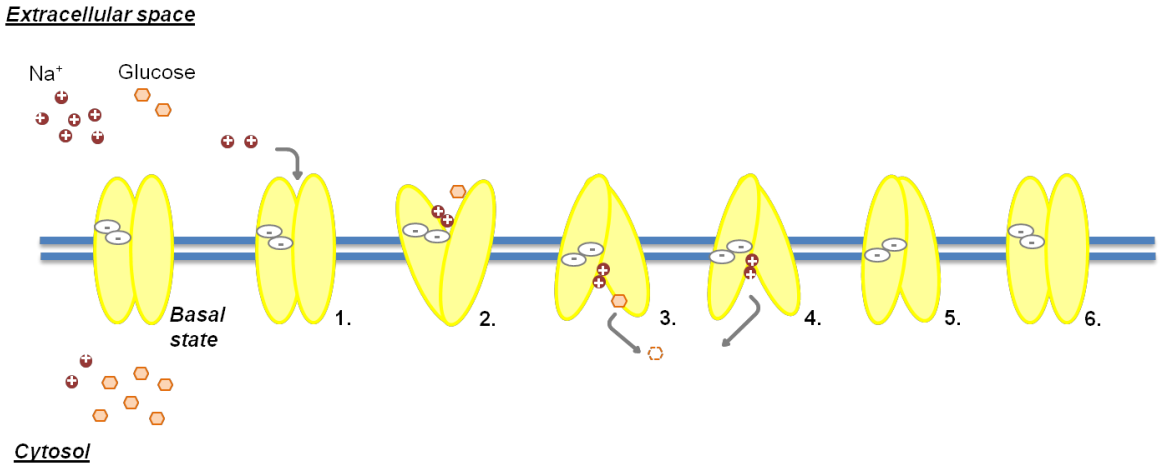


**Figure 7.** Squematic representation of the six-state kinetic model for the $Na^+$/glucose cotransport mediated by SGLT1. The binding of two $Na^+$ ions promotes a conformational change in the cotransporter (1) that allows glucose to bind (2). The substrates are then transported across the membrane where glucose (3) and $Na^+$ dissociate (4). The dissociation results in a relaxation of the protein conformation (5) and the empty sites are then reorientated to the external surface to complete the cycle (6).

In addition, fructose is passively absorbed at the BBM of the intestinal epithelium with the aid of GLUT5 transporter. Afterwards, the absorbed sugars (glucose, galactose and fructose) across the BLM of the enterocyte into the systemic circulation through GLUT2-mediated facilitated diffusion (uniport) (Ferraris and Diamond, 1997; Wright et al., 2003) (**Figure 8**). It has been proposed that GLUT2 is rapidly recruited to the enterocytic BBM when the luminal concentration of glucose or fructose is increased to very high concentration (Affleck et al., 2003; Kellett and Helliwell, 2000). Apical GLUT2 was initially detected in an experimental model of

diabetes but other conditions seem also to lead to this change in glucose transport such as psychological stress (Boudry et al., 2007; Corpe et al., 1996). In addition, some authors believed that the classic model for the intestinal glucose absorption is not valid to explain sugar absorption when the apical SGLT1 and GLUT5 transporters are saturated (Kellett and Brot-Laroche, 2005). However, this apical location of GLUT2 remains a controversial subject to many authors. Evidences from knockout mice and from humans with mutated GLUT2 do not demonstrate the presence of this transporter at the intestinal BBM (Santer et al., 2003; Stümpel et al., 2001). Additionally, immunohistochemical analysis using different GLUT2 antibodies reveals that this transporter is exclusively detected at the BLM of the enterocytes (Dyer et al., 2009; Moran et al., 2010).

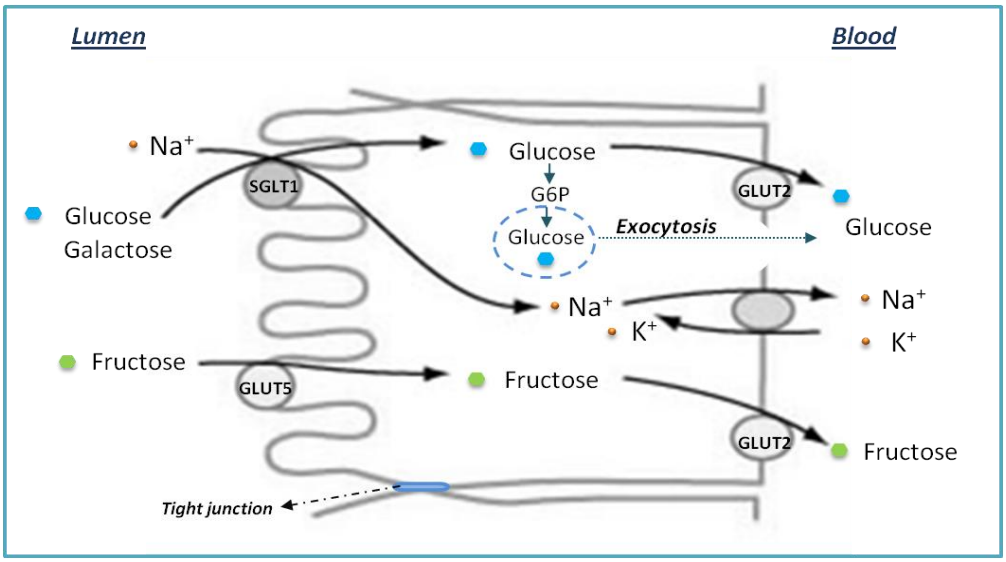


**Figure 8.** Classical model of intestinal sugar transport across an enterocyte. $Na^+$: sodium ions; $K^+$: potassium ions; G6P: glucose-6-phosphate; SGLT1: sodium-glucose cotransporter 1; GLUT5: glucose transporter 5; GLUT2: glucose transporter 2.

A second pathway for the intestinal sugar efflux has been suggested and is not mediated by GLUT2 (Stümpel et al., 2001) (**Figure 8**). The proposed mechanism relies on the fact that part of the glucose that enters in the enterocyte is phosphorylated and the resultant products (glucose-6-phosphate) incorporated into endosomes. Glucose is then delivered into the bloodstream by exocytosis through the BLM (Santer et al., 2003; Wright et al., 2007; Wright et al., 2003).

#### 1.3.1.2. Regulation of intestinal sugar transporters

There are some substances that inhibit the intestinal sugar transporters and thereby repress the intestinal sugar uptake. Dietary phloridzin is a specific and competitive inhibitor of SGLT1 (Boyer and Liu, 2004; Masumoto et al., 2009). The compound is typically found in apples and derived processed foods (such as juice, ciders and purées) (Masumoto et al., 2009; Van Der Sluis et al., 2002). GLUT2 is highly inhibited by phloretin, a product of dietary phloridizin degradation, and cytochalasin B, a cell-permeable fungal toxin (Faria et al., 2009; Ferraris, 2001). D-fructose and L-sorbose derivates showed to repress GLUT5 (Girniene et al., 2003; Miyamoto et al., 1994; Tatibouët et al., 2000).

The expression of sugar transporters is upregulated in experimentally induced diabetes (Azevedo et al., 2011; Burant et al., 1994; Corpe et al., 2001; Corpe et al., 1996). T2DM patients also reveal an enhanced intestinal sugar transport as a result of the increased abundance on intestinal sugar transporters (Douard and Ferraris, 2008; Dyer et al., 2002). In addition, $Na^+/K^+$-ATPase activity is also upregulated in streptozotocin-induced diabetic rats (Azevedo et al., 2011; Drozdowski and Thomson, 2006; Wild et al., 1999).

Luminal carbohydrate content deeply regulates the activity and abundance of the intestinal glucose transporters. Prolonged consumption of high carbohydrate diets enhances SGLT1 expression and leads to increased intestinal sugar absorption (Cheeseman and Harley, 1991; Diamond et al., 1984; Ferraris, 2001; Ferraris and Diamond, 1989; Miyamoto et al., 1993). Furthermore, rats fed with high glucose, galactose or sucrose diets had increased SGLT1 gene expression than those fed on carbohydrate-free or low carbohydrate diets (Ferraris, 2001; Miyamoto et al., 1993; Shirazi-Beechey et al., 1991).

SGLT1 is also regulated by other dietary constituents, including $Na^+$ and fibre. Consumption of a low salt diet showed to be effective in decreasing the activity of SGLT1 due to a depletion of the luminal amount of $Na^+$ (De La Horra et al., 2001; Ferraris, 2001). On the other hand, the effects of dietary fibre on SGLT1 regulation remain inconclusive: while some studies denoted a decrease in intestinal glucose uptake, others revealed that fibre consumption did not modify the intestinal glucose uptake (Ferraris, 2001).

The exact role of insulin on the regulation of these glucose transporters remains poorly understood. In diabetic rats, subcutaneous treatment with insulin showed to restore the normal levels of SGLT1 protein without affecting the mRNA levels of this transporter (Kurokawa et al., 1995). Troglitazone, an insulin sensitizer, showed to reduced SGLT1 expression and the protein levels of GLUT5 in diabetic animals. However, no changes on GLUT2 expression and GLUT5 mRNA levels were observed (Corpe et al., 2001). In Caco-2/TC7, a clone isolated from a late passage of the parental Caco-2 line, insulin targeted sugar absorption by regulating the membrane location of GLUT2 (Tobin et al., 2008). Nevertheless, it has been recently reported that insulin stimulates intestinal glucose transport by increasing the number of SGLT1 transporters and downregulating $Na^+/K^+$-ATPase activity (Serhan and Kreydiyyeh, 2010).

Thus, regulation of these sugar carriers is crucial for glucose homeostasis and has important dietary and clinical implications.

### 1.3.2. The intestinal cholesterol absorption

Despite the undeniable value of the small intestine on sugar absorption and thereby glucose homeostasis, evidence has emerged that this organ is also an important regulator of cholesterol homeostasis.

Animals are capable of producing cholesterol. In small amounts, cholesterol is a crucial compound to the animal's body function: it is a constituent of cell membranes and a precursor for bile acids synthesis and steroid hormones (such as estrogen and androgen) (Chen et al., 2008; Desvergne et al., 2006). Cholesterol has attained a nasty reputation; it is sometimes treated as a "poison" due to its association with CVDs such as atherosclerosis. To avoid the accumulation of high levels of cholesterol, the body has mechanisms that tightly regulate cholesterol metabolism through coordinated effects of dietary absorption, endogenous production and excretion (Dietschy et al., 1993; Schoenheimer and Breusch, 1993). The main sources of cholesterol in the body are the diet (accounting to approximately 60% of the daily cholesterol) and the endogenously synthesized cholesterol, which is influenced by the total amount of dietary cholesterol absorbed (Hui et al., 2008).

In the lumen of the small intestine, dietary cholesterol is included in micelle particles by the action of bile acids to facilitate uptake by the enterocytes. This cholesterol absorption across the BBM of enterocytes is performed by the Niemann-

Pick C1-like 1 (NPC1L1). NPC1L1 activity is inhibited by ezetimibe, a cholesterol absorption inhibitor frequently used in the treatment of hypercholesterolemia (Garcia-Calvo et al., 2005; Spener, 2007). Free cholesterol is taken up by the intestine and esterified in the enterocyte by the action of acyl-CoA:cholesterol acyltransferase 2 (ACAT-2) and then incorporated with triglycerides and apolipoproteins into nascent chylomicrons (Wang, 2007). These lipoproteins will then be secreted into the lymph and afterwards into the bloodstream, where they acquire apo C and apo E from HDL, originating mature chylomicrons (**Figure 9**). (Havel et al., 1973; Sudhop and von Bergmann, 2002; Wang, 2007). The newly attained apo C on the surface of these mature particles activates the lipoprotein lipase (LPL), an endothelial enzyme, that hydrolyze their triglyceride core into FFA and glycerol that will be taken up by several tissues (Bengtsson and Olivecrona, 1980; Jackson et al., 1980; Warnakula et al., 2011).

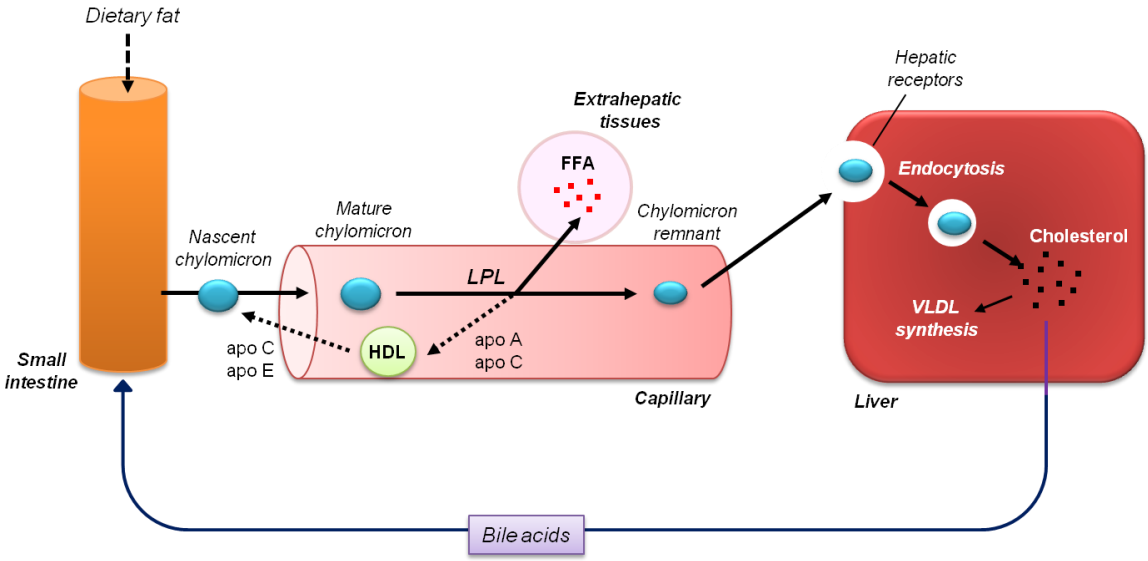


**Figure 9.** Metabolic fate of chylomicrons. Apo: apolipoproteins (A, C and E); HDL: high-density lipoprotein; LPL: lipoprotein lipase; FFA: free fatty acid; VLDL: very low-density lipoprotein; hepatic receptors: LDL receptor (LDLR) or LDL receptor-related protein (LRP).

At the same time, most of the apo A and apo C particles are transferred to HDL and the chylomicrons are converted into smaller particles, the chylomicrons remnants. These particles, relatively enriched in cholesterol, apo B48 and apo E, will be removed from circulation after interaction with specific apo E receptors in the liver (including LDL receptor, [LDLR] and LDL receptor-related protein, [LRP]) (Hui et al., 1981; Redgrave and Small, 1979; Sherrill et al., 1980; Warnakula et al., 2011). Once endocytosed, the remnant chylomicrons are degraded within the lysosomes and their

constituents released. These summarized stages constitute the exogenous pathway of lipid transport (**Figure 9**).

Otherwise, a large amount of the absorbed cholesterol returns to the intestinal lumen for biliary cholesterol excretion, a process mediated by the brush-border ATP-binding cassette transporters ABCG5/ABCG8 (Wang, 2007; Yu et al., 2002b; Yu et al., 2002c). A small amount of cholesterol that enters the enterocyte is incorporated in nascent apo AI particles, the rate-controlling step in HDL formation. This process is mediated by the ATP-binding cassette transporters A1 (ABCA1) located at the BLM (Beltowski, 2008; Wellington et al., 2002).

Thus, the intestinal cholesterol absorption is a complex process that involves and is regulated by several genes. The liver X receptor (LXR), is probably the major regulator of cholesterol metabolism since it regulates multiple genes involved in cholesterol efflux (like ABCA1, ABCG5, ABCG8) (Repa et al., 2002; Repa et al., 2000b; Venkateswaran et al., 2000), bile acid synthesis (cholesterol-7α-hydroxylase, [Cyp7α1]) (Lehmann et al., 1997; Peet et al., 1998) and lipogenesis (SREBP-1c, FAS, ACC) (Repa et al., 2000a). Therefore, LXR activation is a "two-edged knife": on one hand, it protects cells against cholesterol overload by inhibiting cholesterol absorption, stimulating cholesterol efflux to the liver and its conversion into bile for biliary excretion (Beltowski, 2008). On the other hand, LXR agonists also showed to induce hypertriglycemia (elevated plasma triglyceride levels) and hepatic steatosis (fatty liver) by enhancing hepatic lipogenesis (Talukdar and Hillgartner, 2006; Yoshikawa et al., 2001).

Therefore, the small intestine should be considered as an emerging target for the development of novel strategies that helps to maintain both glucose and lipid homeostasis and thereby preventing pathogenic conditions.

### 1.3.3. The *in vitro* intestinal models HT-29 and Caco-2 cells

Attention has turned to the use of human cell culture systems as an alternative to time-consuming and resource-intensive *in vivo* studies. Human colonic HT-29 and Caco-2 cells have been widely used in many areas of pharmacology and toxicology

research, since they display a number of properties characteristic of differentiated intestinal cells (Gan et al., 1994; Hidalgo et al., 1989; Huet et al., 1987).

Under standard culture conditions, HT-29 cells form a non-polarized, undifferentiated multilayer under (Cohen et al., 1999; Simon-Assmann et al., 2007). However, in specific culture conditions, these cells form polarized and differentiated monolayers that express typical enterocyte brush-border enzymes and well-developed tight junctions (Huet et al., 1987; Simon-Assmann et al., 2007). Among other experimental approaches, butyrate has been reported to induce HT-29 differentiation instead of growth (Archer et al., 1998; Barnard and Warwick, 1993; Hodin et al., 1996). Butyrate is a short-chain fatty acid resultant from the colonic bacterial fermentation of dietary carbohydrate and fibres (Topping and Clifton, 2001). Two mechanisms have been proposed for the butyrate-mediated inhibition of cell growth: one is related with histone hyperacetylation and p21 induction (that leads to cell cycle arrest) and the other involves decreased responsiveness to the epidermal growth factor (Archer et al., 1998).

Kinetic studies showed that differentiated HT-29 cells contain a $Na^+$-dependent sugar transporter sensitive to phloridzin (SGLT1) (Blais, 1991). In addition, it has been reported that the carbohydrate metabolism of cultured HT-29 cells responds to insulin (Babia et al., 1989). These findings indicate that HT-29 cells are valuable models for studying the regulation of intestinal sugar transport, but also the *in vitro* mechanisms of insulin action.

Caco-2 cells differentiate spontaneously in culture and acquire structural and functional characteristics that resemble mature intestinal enterocytes (Engle et al., 1998; Pignata et al., 1994; Pinto et al., 1983). These cells reach confluency in 2-3 days and the stationary growth phase after 10 days in culture, while differentiation occurs within 20 days (Braun et al., 2000; Pinto et al., 1983). Differentiated Caco-2 cells express typical brush-border hydrolases such as intestinal alkaline phosphatase (ALP) and sucrase-isomaltase and form a polarized monolayer connected by well-developed tight junctions (Engle et al., 1998; Matsumoto et al., 1990; Ranaldi et al., 2003; Simon-Assmann et al., 2007). Since Caco-2 cells express sugar transporters such as the ones found in the small intestine, they are considered valuable *in vitro* models for studying the activity and regulation of these transporters (Chang et al., 2007; Harris et al., 1992; Mahraoui et al., 1994). Kipp and colleagues (2003) reported that, in Caco-2, SGLT1 is located in intracellular compartments that are associated with microtubules. Therefore, the authors considered that the intracellular pool of SGLT1 is highly mobile and the microtubules

function as the "railroad tracks" for the intracellular SGLT1 trafficking (Kipp et al., 2003). Chang and colleagues (2007) demonstrated that specific ginsenosides modulates intestinal glucose uptake through modifying SGLT1 expression in Caco-2 cells.

However, HT-29 and Caco-2 cells possess characteristics that resemble their colonic origin and also lack different cell types observed in the intestinal epithelium of animals and humans (Engle et al., 1998; Hilgendorf et al., 2000; Pontier et al., 2001; Rubas et al., 1993). Caco-2 cells are described to possess lower permeability than the human small intestine for hydrophilic compounds that across the epithelium and for compounds that are transported into the cell by specific carriers (Hilgendorf et al., 2000). In order to obtain a more reliable *in vitro* model for the human small intestine, several approaches have been tried, including the search and isolation of new HT-29 or Caco-2 clones with improved characteristics or even the establishment of co-cultures of both cell lines (Hilgendorf et al., 2000; Pontier et al., 2001; Thomson et al., 1997).

Despite presenting several limitations, both HT-29 and Caco-2 cells are still extensively used to study a wide range of issues ranging from the search of new drugs with beneficial pharmacologic effects, to the mechanisms intrinsic to the development of the intestinal epithelium and cell function (Simon-Assmann et al., 2007).

## 1.4. The liver: some morphological and functional aspects

The liver is the biggest solid organ of the human body and is responsible for multiple metabolic functions which include 1) the removal of xenobiotics, metabolites and other foreign molecules; 2) the synthesis and release into the blood of molecules that support whole body homeostasis (such as glucose and plasma proteins); 3) the synthesis of bile and its excretion into the intestine and 4) the storage of several substances such as glycogen, fat and vitamins (Ramadori et al., 2008).

The cellular architecture of the liver is composed by distinct and well organized sub-populations: the parenchymal and non-parenchymal cells. The highly differentiated parenchymal cells, also known as hepatocytes, are the most abundant (representing 70 to 80% of all) and the main functional cells in the liver (Ramadori et al., 2008). Hepatocytes play an important role on protein synthesis and storage, carbohydrate and lipid metabolism as well as in detoxification and excretion of substances. The main non-parenchymal hepatic cells (Kupffer cells, sinusoidal cells and steallate cells) are

responsible for regulating hepatocyte proliferation and modulating liver structure (Malik et al., 2002).

Like the small intestine (see section 1.3), the liver has also critical physiological functions in maintain both glucose and lipid homeostasis. This section will introduce some of the main intervenients of hepatic glucose and lipid metabolism.

### 1.4.1. Hepatic glucose metabolism and its regulation

In response to the body' shifting demands, blood glucose levels are tightly maintained by hepatocytes through a process called gluconeogenesis (Klover and Mooney, 2004). This is mainly performed by the liver although, to a lesser extent it could also occur in the kidney, and is vital for normalizing glucose supply to the central nervous system. Starvation stimulates the production of glucagon by the pancreatic α-cells (**Figure 3**, see section 1.2) and its secretion into circulation in order to increase glycaemia, by stimulating hepatic glucose production and subsequent release (Jiang and Zhang, 2003). Once released in the bloodstream, glucagon binds to its hepatic receptor and activates adenylate cyclise, which increases cyclic adenosine monophosphate (cAMP) levels. This increase induces the cAMP-dependent protein kinase (PKA) that stimulates the expression of gluconeogenic genes (like PEPCK and G6Pase) and increases gluconeogenesis (Agius, 2007). PKA is also responsible for inhibiting glycogenesis (through the inactivation of glycogen synthase) and glycolisis (by repressing glycolitic genes like piruvate kinase) (Agius, 2007; Jiang and Zhang, 2003).

The increased levels of glucose after feeding inhibit glucagon secretion and stimulate insulin production and release by the pancreas. Insulin stimulates the hepatic glycogen synthesis, by activating the PI3K/Akt cascade, and blocks the hepactic glycogenolysis and gluconeogenesis (Aronoff et al., 2004; Lee and Kim, 2007). However, the hepatic glucose uptake is not directly stimulated by insulin. Postprandial glucose levels are cleared from circulation through the GLUT2 transporter and the sugar is rapidly converted in glucose-6-phosphate by glucokinase (Bae et al., 2010)

Gluconeogenesis is regulated by AMP-activated protein kinase (AMPK), a major energy sensor and a regulator of the cellular energy homeostasis (Carling, 2004; Kahn et al., 2005; Lage et al., 2008). Several effectors have been described to induce AMPK activation ranging from hypoxia, exercise, and nutrient deprivation, that increases

AMP:ATP ratio to modifications of intracellular $Ca^{2+}$ levels (Hardie, 2004; Kahn et al., 2005; Zhang et al., 2009). AMPK is also activated by medicines such as metformine and thiazolidinedione and by a specific activator the adenosine analog 5-aminoimidazole-4-carboxamide riboside (AICAR) (Carling, 2004; Fryer et al., 2002). Activated AMPK induces phosphorylation of multiple downstream targets to restrain energy-consuming pathways (such as hepatic gluconeogenesis and lipogenesis) and support energy-producing processes (like lipolysis and glycolysis), in order to re-establish energy balance (Carling, 2004; Hardie et al., 2003; Long and Zierath, 2006; Zhang et al., 2009).

Thus, the decision of activating glucose production when nutrient supply is limited, or to store glucose into glycogen when nutrient delivery is abundant, makes the liver a "buffering" system (Desvergne et al., 2006).

### 1.4.2. Hepatic lipid metabolism: the role of SREBP

When energy intake is abundant, the liver has also the ability to convert glucose into triglycerides through *de novo* synthesis, following the restore of the hepatic glycogen levels (Reddy and Hashimoto, 2001). This lipogenic pathway is stimulated by glucose in three different ways: 1) by serving as a substrate to fatty acid synthesis; 2) by inducing the transcription of lipogenic genes through the activation of carbohydrate responsive element-binding protein (ChREBP) and 3) by stimulating the pancreatic production/secretion of insulin instead of glucagon (Kersten, 2001).

The recent identification of transcription factor ChREBP shed some light on the potential mechanism whereby glucose controls lipogenic gene transcription. Under low glucose concentrations, ChREBP is phosphorylated and localized in the cytosol of hepatocytes. Although under elevated glucose levels, ChREBP is rapidly translocated into the nucleus (Dentin et al., 2005; Uyeda et al., 2002). This nuclear translocation is regulated by dephosphorylation and phosphorylation events (Dentin et al., 2006; Postic et al., 2004). Once in the nucleus, ChREBP binds to glucose- or carbohydrate-response element (ChoRE) and activates glycolytic (like liver-type pyruvate kinase, [L-PK]) and lipogenic (as FAS and ACC) gene expression (Dentin et al., 2005; Kawaguchi et al., 2001).

An additional pathway for the transcriptional activation of genes related with glucose and lipid metabolism has been proposed: the SREBP pathway. The SREBP family comprises three members in mammals with distinct tissue distribution: SREBP-1a, SREBP-1c (both encoded from a single gene) and SREBP-2 (encoded by a different gene) (Amemiya-Kudo et al., 2002; Desvergne et al., 2006; Osborne, 2000). SREBP-1a is predominantly expressed in cultured cell lines, while SREBP-1c and SREBP-2 are predominant expressed in several tissues such as the liver (Desvergne et al., 2006; Horton et al., 2002; Shimomura et al., 1997). SREBP-1c regulates the expression of genes involved in fatty acids, triglycerides and phospholipids synthesis (such as ACC and FAS), whereas SREBP-2 controls the transcription of cholesterolgenic genes (like the 3-hydroxy-3-methylglutaryl coenzyme A reductase [HMGCR] and the LDLR) (Brown and Goldstein, 1997; Horton et al., 2002). SREBP-1a isoform appears to activate the transcription of genes involved in both pathways (Amemiya-Kudo et al., 2002; Eberle et al., 2004; Inoue et al., 2005).

After translation, SREBPs remained anchored to ER membranes. They are associated to SREBP cleavage activating protein (SCAP) that in turns binds to the insulin-inducible gene (Insig), which retains SCAP/SREBP complexes within the ER compartment (**Figure 10**). Upon stimulation, such as a decrease in sterol levels, the affinity of SCAP and Insig decrease, leading to protein dissociation and SREBP is escorted by SCAP into the Golgi apparatus (Goldstein et al., 2002). In this organelle, SREBP is proteolytically cleaved by the action of two distinct proteases (site 1 protease, [S1P] and site 2 protease, [S2P]), releasing the N-terminal of SREBP that migrates to the nucleus (Horton et al., 2002; Hughes et al., 2005; Yang et al., 2002) (**Figure 10**). After released, the SCAP-free Insig fragment is rapidly degraded by ubiquitination (Raghow et al., 2008). Once in the nucleus, the N-terminal of SREBP (also called nuclear SREBP) binds to the sterol response elements (SREs) in the promoter region of target genes and induces their transcription (Raghow et al., 2008) (**Figure 10**). As intracellular sterol levels increase, SCAP/SREBP triggers a conformational change that allows the complex to bind to Insig, maintaining the stable complex in ER (Osborne, 2000; Yang et al., 2002).

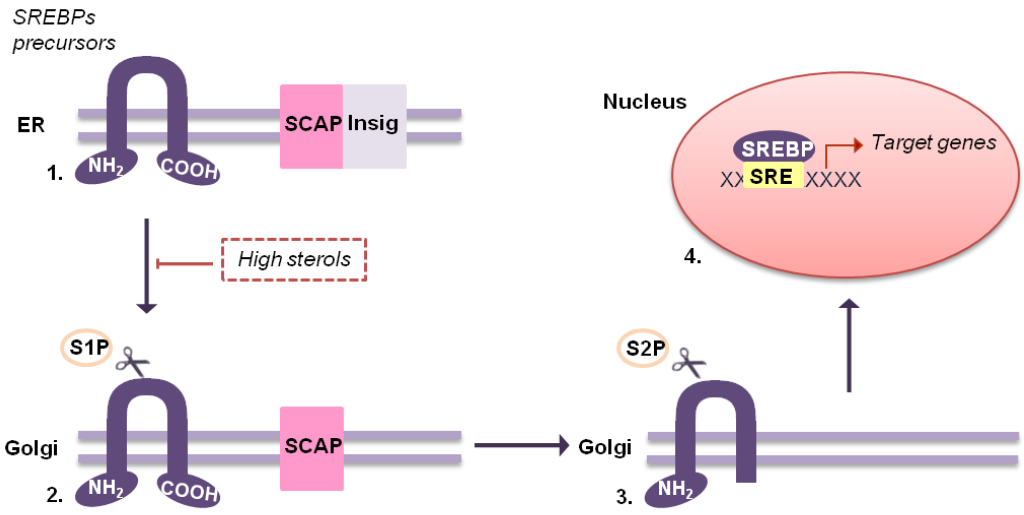


**Figure 10.** SREBPs activation throught proteolytic cleavage. Inactive precursors of SREBPs are retained in ER, tightly associated with SCAP protein, which interacts with Insig proteins (1). Under stimulation, SREBPs are escorting to the Golgi apparatus by SCAP protein, and cleaved by S1P at the luminal site (2). S2P promotes a second cleavage which leads to SREBPs release (3) and consequent translocation to the nucleus, where SREBPs bind on the SREs in the promoter region of their target genes (4). This pathway is repressed by elevated sterol levels. SREBP: sterol regulatory element binding protein; $NH_2$: amino-terminal; COOH: carboxyl-terminal; SCAP: SREBP cleavage activating protein; Insig: insulin-inducible gene; ER: endoplasmic reticulum; SRE: sterol regulatory element; S1P/S2P: site proteases.

In addition to proteolytic cleavage, SREBPs are also regulated by transcriptional events. SREBP-1c expression is regulated by the LXRα, which is activated by oxysterols and other derivates of cholesterol metabolism (Desvergne et al., 2006; Janowski et al., 1999; Lehmann et al., 1997). LXRα attaches and activates to the SREBP-1c promoter region, inducing gene transcription (Repa et al., 2000a; Wong and Sul, 2010). LXRα$^{-/-}$ animals showed reduced basal levels of SREBP-1c, FAS and ACC (Repa et al., 2000a; Schultz et al., 2000).

The transcriptional activation of SREBP-1c by insulin has been demonstrated in hepatocytes, adipocytes and skeletal muscle (Ducluzeau et al., 2001; Foretz et al., 1999a; Hegarty et al., 2005; Kim et al., 1998). The metabolic effects of insulin in SREBP-1c expression are mediated by the activation of multiple downstream effectors of the insulin signalling pathway. The insulin-induced activation of PI3K pathway leads to stimulation of both Akt and atypical PKC, which in turns increase SREBP-1c expression (Farese et al., 2005; Matsumoto et al., 2003; Taniguchi et al., 2006). This insulin-induced SREBP-1c expression is counteracted by glucagon as well as by AMPK activation (Foretz et al., 1999b; Zhou et al., 2001). Studies performed mainly in cultured cells, demonstrated that insulin also stimulates the transcriptional activity of nuclear

SREBP-2 and SREBP-1a, by the activation of the MAPK pathway (Kotzka et al., 2004; Roth et al., 2000).

SREBP-1c and SREBP-2 have been associated with fatty liver and insulin resistance. Obese mice with insulin resistance and hyperinsulinemia revealed increased SREBP-1c levels in their fatty livers (Shimomura et al., 1999). Insulin resistant obese mice showed a reduction on hepatic nuclear SREBP-1c and lipid storage, after metformin treatment (Lin et al., 2000; Zhou et al., 2001).

Thereby, the knowledge of the transcriptional and posttranscriptional mechanisms that regulates SREBP constitutes an important issue for disease prevention.

#### 1.4.2.1. Fatty acid metabolism and regulation

The biosynthesis of fatty acids, also called *de novo* synthesis, is a process managed by two key enzymes, the ACC and FAS that among other factors, are regulated by SREBP-1.

ACC comprises two major isoenzymes in humans and other animals that are encoded by distinct genes and display different functions, regulation and subcellular distributions (Abu-Elheiga et al., 2000; Cronan and Waldrop, 2002; Tong and Harwood, 2006). Although ubiquitous expressed, ACC1 (ACCα, 265 kDa) is highly found in lipogenic tissues such as the liver and adipose tissues. On the other hand, the ACC2 (ACCβ, 280 kDa) is found in oxidative tissues such the liver, heart and skeletal muscle (Abu-Elheiga et al., 2000; Iverson et al., 1990; Kim, 1997). ACC1 is responsible for the hepatic production of malonyl-CoA that will be shuttled for fatty acid synthesis and elongation, resulting in triglyceride and VLDL synthesis. The conversion of acetyl-CoA and malonyl-CoA substrates into palmitate is conducted by FAS, a complex multifunctional enzyme. FAS is found in lipogenic tissues such as the liver and adipose tissue (Latasa et al., 2000; Liu et al., 2010).

ACC2 also produced malonyl-CoA, which will regulate fatty acid oxidation by modulating the activity of carnitine palmitoyltransferase 1 (CPT1) (Brownsey et al., 2006; McGarry and Brown, 1997; Tong and Harwood, 2006). CPT1 is located on the outer mitochondrial membrane and regulates the transport of long chain fatty acyl-CoAs into the mitochondria to be oxidized (Kerner and Hoppel, 2000; Stanley et al., 2005). CPT1 catalyzes the production of acylcarnintine, which is transported by carnitine translocase across the inner mitochondrial membrane. Acylcarnintine is then

reconverted to long chain fatty acyl-CoAs via carnitine palmitoyltransferase 2 (CPT2), which enters in the β-oxidation pathway for energy production (McGarry and Brown, 1997; Zhang et al., 2010). This shuttle system is inhibited by malonyl-CoA substrate. Thus, malonyl-CoA is a key substrate in fatty acid metabolism, since it controls the switch between fatty acid synthesis and oxidation.

Both ACC isoforms are regulated by dietary and hormonal states. ACC1 and ACC2 expression and activity is upregulated in animals submitted to a carbohydrate-rich, low-fat diet and dowregulated in starvation and diabetes (Abu-Elheiga et al., 2000; Kim, 1997). The later effects on ACC1 can be reverted by submitting these animals to treatment with insulin (Abu-Elheiga et al., 2000; Kim, 1997). In fact, insulin and glucose reduces ACC1 phosphorylation and stimulates its activity, contrarily to glucagon (Kim, 1997; Saha and Ruderman, 2003; Witters et al., 1988). Upon activation, AMPK phosphorylates and inactivates both ACC isoforms, which reduces malonyl-CoA intracellular levels. This decreases fatty acid synthesis and activates fatty acid oxidation via stimulation of CPT1 (Kahn et al., 2005; López et al., 2008; Winder et al., 1997; Wolfgang and Lane, 2006). As afore mentioned, ACC and FAS expression is regulated by SREBP-1, LXR and ChREBP transcription factors (Barber et al., 2005; Brownsey et al., 2006; Shimano, 2000).

FAS expression is also regulated by dietary and hormonal status: it is stimulated by carbohydrate ingestion, thyroid hormone and insulin and inhibited by unsaturated fatty acids, cAMP and glucagon (Lakshmanan et al., 1972; Paulauskis and Sul, 1989; Soncini et al., 1995; Sul and Wang, 1998). Treatment with pu-erh tea, a post-fermented tea produced in China, reduced the hepatic expression of FAS in rats and HepG2 cells through downregulation of the JNK and PI3K/Akt signaling cascades (Chiang et al., 2005).

CPT1 expression is highly regulated by a member of the peroxisome proliferator activated-receptor family, the PPARα (Minnich et al., 2001). PPARα induces fatty acid consumption, cholesterol catabolism and controls lipoprotein assembly (Lefebvre et al., 2006; Reddy and Hashimoto, 2001; Staels et al., 1995). In addition, PPARα also attenuates inflammation and decreases plasma triglyceride levels and adiposity, which prevents CVDs and insulin resistance (Berger et al., 2005; Guerre-Millo et al., 2000; Lefebvre et al., 2006). Numerous molecules are described to act as PPARs activators such as the dietary fatty acids (especially the unsaturated fatty acids) and oxidized

phospholipids (Chinetti-Gbaguidi et al., 2005). In clinical practice, PPARα is activated by fibrates (Gervois et al., 2007; Lee et al., 1995; Lehmann et al., 1995).

Thereby, modified fatty acid metabolism contributes to dyslipidemia and insulin resistance, features of several metabolic disorders. The modulation of fatty acid metabolism may provide a feasible therapeutical approach for preventing disease progression and associated morbidities.

#### 1.4.2.2. The hepatic cholesterol *de novo* synthesis

As afore mentioned (see section 1.3.2), mammalian cells obtain cholesterol mainly from two sources: the diet and the endogenous synthesis. Despite roughly all cells possess the ability to synthesize cholesterol, the liver arises as the major manufacturer of cholesterol since it produces as much cholesterol as the extrahepatic tissues jointed (Dietschy et al., 1993). The hepatic cholesterol biosynthesis is a process tightly regulated by the amount of dietary cholesterol that reaches the liver from the intestine through the chylomicron remnant pathway (Dietschy et al., 1993). The rate limiting enzyme in the cholesterol synthetic pathway is the HMGCR, which catalyzes the conversion of HMG CoA into mevalonate and is a target of statins (Jasinska et al., 2007). Cholesterol is master regulator of HMGCR enzyme. High cholesterol levels lead to either a decrease in HMGCR activity, by directly inducing ubiquitination and degradation of the enzyme and to a blockade of the SREBP-2 activation that results in decreased HMGCR gene expression. Cholesterol abundance is also a repressor of transcriptional LDLR activation-mediated by SREBP-2, leading to a blockage in cholesterol uptake from plasma LDL (Berg et al., 2002). Thus, the hepatic cholesterol *de novo* synthesis and LDL uptake are rapidly down-regulated when relatively large amounts of cholesterol reach the liver.

Conversely, cholesterol-lowering treatments with statins, competitive inhibitors of HMGCR, reduced the amount of cholesterol produced by the liver, leading to SREBP-2 processing, which increases the number of LDLR displayed on liver surface (Endo et al., 1977; Goldstein and Brown, 2009; Tobert, 2003). These receptors promote LDL and LDL precursor's clearance from the circulation and consequently reduce the associated health risks of high cholesterol circulating levels (Brown and Goldstein, 1986; Twisk et al., 2000).

The healthy liver is prepared for managing large amounts of cholesterol by 1) converting cholesterol into cholesterol esters (CEs) via ACAT2, which are stored in cytosolic lipid droplets to be used when occasion may require (Kruit et al., 2006); 2) by producing and secreting VLDL particles (Kruit et al., 2006) and 3) by converting cholesterol into bile acids that enters the small intestine where it is further reabsorbed (enterohepatic circulation) or excreted into feces (Ikonen, 2008).

#### 1.4.2.2.1. Cholesterol efflux

Like the small intestine, the liver is capable to produce and secrete another class of lipoproteins, the VLDL particles. The hepatic free cholesterol is packaged with triglycerides and apolipoproteins (apo B100, E and C) to form the nascent VLDL, which are released directly into bloodstream. Similar to chylomicrons, nascent VLDL particles are converted into mature VLDL after achieving additional apo E and apo C II from HDL (Costet, 2010; Nestruck and Rubinstein, 1976; Swift et al., 1980).

Following hydrolysis of the triglyceride content, the released fatty acids are absorbed in the extrahepatic tissues and apo C particles are transferred to HDL (Mahley et al., 1984; Patsch et al., 1978; Wang and Eckel, 2009). This result in a progressive shrinkage of mature VLDL to form remnant particles quite enriched in apo B100 and apo E, the intermediate-density lipoprotein (IDL) (**Figure 11**). Some of these particles can be directly removed from circulation through interaction with hepatic lipoprotein receptors (LDLR and LRP), a process that requires apo E (Kita et al., 1982; Masson et al., 2009; Stalenhoef et al., 1986). The remaining IDL particles that are not removed from circulation are further hydrolyzed by the hepatic lipase (HL) to form small dense LDL (Chappell and Medh, 1998; Mudd et al., 2007). LDL is frequently considered the "bad cholesterol", due to its positive association with atherosclerosis. LDL has relatively little triglyceride but high cholesterol content, holding only apo B100 from the "delipidation cascade" of VLDL to LDL (Avramoglu et al., 2006; Scott et al., 1987). The cellular uptake and catabolism of LDL requires the interaction of its apo B100 and the LDLR on plasma membranes of hepatocytes and extrahepatic cells (such as vascular smooth muscles and lymphocytes) (Goldstein et al., 1983; Twisk et al., 2000). Afterwards, LDL particles are internalized, degraded by lysosomal enzymes, and cholesterol esters are transformed in cholesterol and fatty acids (**Figure 11**). This

cholesterol can be further used for 1) membrane synthesis or steroid hormone synthesis; 2) repressing the *de novo* cholesterol synthesis, by inhibiting the activity of the HMGCR; 3) activating the intracellular ACAT enzyme, for cholesterol esterification and 4) decreasing LDLR in the cellular surface, which increase cholesterol content in bloodstream (Avramoglu et al., 2006; Goldstein and Brown, 1977).

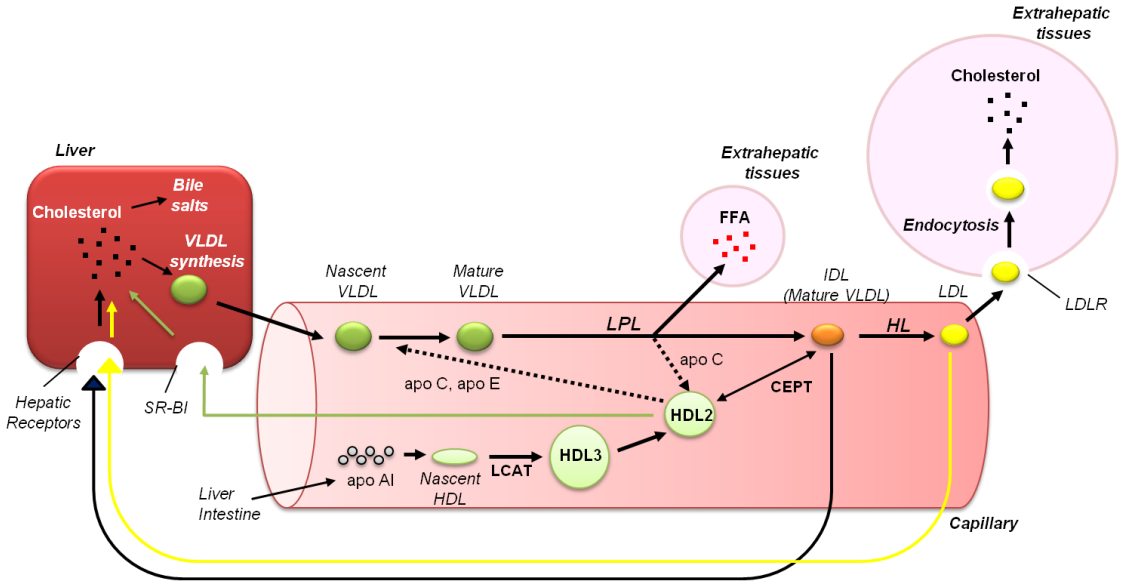


**Figure 11.** Schematic diagram of the endogenous route for lipid transport. VLDL: very low-density lipoprotein; apo: apolipoproteins (AI, C and E); LPL: lipoprotein lipase; FFA: free fatty acid; IDL: intermediate-density lipoprotein; HL; hepatic lipase; LDL: low-density lipoprotein; HDL: high-density lipoprotein; hepatic receptors: LDL receptor (LDLR) or LDL receptor-related protein (LRP); SR-BI: scavenger receptor class B type I; LCAT: lecithin-cholesterol acyltransferase; CETP: cholesterol transfer protein.

The last class of lipoproteins are the well reputed HDL. These lipoproteins serve an important scavenger function in removing the excess of cholesterol from the extrahepatic tissues back to the liver for excretion, the usually called reverse cholesterol transport (Ohashi et al., 2005). The apolipoproteins that compose HDL are mainly secreted by the intestine and the liver. Once released into the bloodstream, these apolipoproteins contact with phospholipids, cholesterol and other apopolipoproteins released from chylomicrons and VLDL, originating nascent disk shaped HDL (Marshall and Bangert, 2004). Nascent HDL collects free cholesterol from cellular membranes or from macrophages and foam cells. The absorbed cholesterol is then esterified by the enzyme lecithin-cholesterol acyltransferase (LCAT), which is activated by apo AI (Patsch et al., 1978; Rye et al., 1999; Santamarina-Fojo et al., 2000). As the produced cholesteryl esters occupy the lipid core of the particle, discoidal HDL is converted into spherical small dense HDL3 (**Figure 11**). Further supply of free cholesterol followed by

cholesterol esterification converts HDL3 into larger and less dense particles, HDL2 (Hamilton et al., 1976; Tabet and Rye, 2009; Warnick et al., 2001). An amount of the HDL-cholesteryl esters may also be transferred to other apo B-containing lipoproteins in exchange for triglyceride, a process mediated by cholesterol transfer protein (CETP). HDL-cholesteryl esters enter the liver through a mechanism mediated by the scavenger receptor class B type I (SR-BI), which involves HDL dissociation at the surface of hepatocytes (van der Velde, 2010). The lipid-depleted HDL particles return to circulation, where it continues to attract cholesterol from cellular membranes, macrophages and foam cells (Byers, 1964; Tabet and Rye, 2009). Thus, through reducing the accumulation of cholesterol in the artery wall, HDL prevents the incidence of atherosclerosis, reason why they are often considered "the good cholesterol".

Imbalances between circulating levels of LDL relatively to those of HDL lead to atherogenesis development. Circulating LDL are highly susceptible to undergo oxidation in the arterial wall through several mechanisms that include free-radical generation and enzymatic activity (Heinecke, 1998; Mertens and Holvoet, 2001). Once oxidized, LDL are retained in the artery wall and induce an inflammatory response that ultimately leads to atherosclerotic plaque formation (Carmena et al., 2004; Lusis, 2000). Unstable atherosclerotic plaques block blood arteries, causing strokes and heart attacks, the leading lethal CVD (Heinecke, 1998; Lusis, 2000).

Since CVDs are associated with multiple metabolic disorders, including T2DM and MetS, pronounced changes in lifestyle in combination with new therapeutic interventions are required to prevent pathological events.

## 1.5. Therapeutical approaches in T2DM

### 1.5.1. Pharmacological interventions

The increased number of diabetic people demands urgent action on early treatment and prevention. Several studies reported that individuals at high risk to develop diabetes can delay or prevent disease progression through lifestyle changes (intensive diet and increased physical activity) and pharmacological interventions (Chiasson et al., 2002; Knowler et al., 2002; Tuomilehto et al., 2001).

Although nonpharmacologic therapy remains a critical component in prevention and management of T2DM, pharmacological therapy is often required to achieve optimal glycaemic control. The number of available oral pharmacological agents has increased significantly in the last years and several new others are being tested for further approval (**Table 2)**. Each class of oral agent has advantages and disadvantages and their proper selection requires a full understanding of their mechanisms of action, associated side effects and patient-specific characteristics.

**Table 2**. Oral agents used to improve glycaemic control. Adapted from [Cheng and Fantus, 2005; Ripsin et al., 2009].

| Drug class | Outcome | Target site | Mechanisms of action | Side effects | Specific agent |
|---|---|---|---|---|---|
| **Sulfonylureas** | Insulin secretion enhancement | β-cells (sulfonylurea receptor) | β-cell membrane depolarization | Hypoglycaemia<br>Weight gain | Glicazide<br>Glimepiride<br>Glibenclamide (Glyburide) |
| **Non-sulfonylureas** | Insulin secretion (brief) stimulation | β-cells (sulfonylurea receptor) | β-cell membrane depolarization | Hypoglycaemia (lower risk than sulfonylureas)<br>Weight gain | Nateglinide<br>Repaglinide |
| **Biguanidines** | Insulin sensitivity improvement | Hepatic/ peripheral tissues (muscle) | Lipid and glucose metabolism regulation (via AMPK activation)<br>Hexokinase expression stimulation | Bloating<br>Abdominal discomfort<br>Diarrhoea<br>Lactic acidosis (rare) | Metformin |
| **α-Glucosidase inhibithors** | Carbohydrate absorption prevention | Gastrointestinal tract | Digestive brush-border enzymes inhibition | Bloating<br>Abdominal discomfort<br>Diarrhoea<br>Flatulence | Acarbose<br>Miglitol |
| **Thiazolidinediones** | Insulin sensitivity improvement | Hepatic/ peripheral tissues (muscle, adipocytes) | Lipid and carbohydrate metabolism regulation (PPARγ agonist) | Weight gain<br>Edema<br>Anemia | Troglitazone<br>Pioglitazone<br>Rosiglitazone |
| **Intestinal lipase inhibitors** | Intestinal fat absorption decline | Intestine | Gastric and pancreatic lipases inhibition | Weight loss<br>Flatulence<br>Increased defecation | Orlistat |

Since T2DM is a progressive disease, most of the times patients will require combination therapy with a second oral agent to assure better glycaemic control

(DeFronzo, 1999; United Kingdom Prospective Diabetes Group, 1998). Although, if these approaches fail, insulin therapy must be initiated immediately in order to achieve satisfactory control (DeFronzo, 1999).

In addition, the management of diabetic dyslipidemia becomes a pivotal approach for the cardiovascular prevention of T2DM patients. The avaiable lipid-lowering therapy comprises several pharmacological drugs that act to maintain lipid homeostasis (**Table 3**).

**Table 3.** Pharmacologic agents used in the management of dyslipidemias. Adapted from [Hachem and Mooradian, 2006; Manninen et al., 1988; Mooradian, 2009; Toth, 2005].

| Drug class | Mechanism of action | Outcome | Efficacy | Side effect | Specific agents | Contra-indications |
|---|---|---|---|---|---|---|
| **Statins** | HMGCR inhibition | ↓ Cholesterol synthesis<br>↑Hepatic LDL clearance | ↓ LDL (18-55%)<br>↑ HDL (5-15%)<br>↓TG (7-30%) | Hepatotoxicity<br>Myophaty | Lovastin<br>Pravastatin<br>Fluvastatin<br>Simvastatin<br>Atorvastatin<br>Rosuvastatin | Liver disease<br>Pregnancy<br>Breast-feeding,<br>Certain drugs[1] |
| **Cholesterol absorption inhibitors** | NPC1L1 inhibition | ↓ Sterol transport across BBM | ↓ LDL (15-20%)<br>↑ HDL (1%)<br>↓TG (8%) | No major adverse effects | Ezetimibe | None |
| **Nicotinic acid (niacin)** | Cholesterol reduction<br>HDL induction | ↓ Hepatic TG formation<br>↓ LDL synthesis<br>↑ HDL synthesis | ↓ LDL (5-25%)<br>↑ HDL (15-35%)<br>↓TG (20-50%) | Hot flashes<br>Hyperglycaemia,<br>Hyperuricemia,<br>Hepatotoxicity | Niaspan<br>Slo-niacin<br>Enduracin | Liver disease<br>Peptic ulcer disease |
| **Fibrates** | PPARα activation | ↓ VLDL formation<br>↓ TG synthesis<br>↑ Fatty acid catabolism<br>↑HDL levels | ↓ LDL (5-20%)<br>↑ HDL (10-15%)<br>↓TG (20-30%) | Dyspepsia,<br>Gallstones,<br>Hepatotoxicity,<br>Myopathy | Clofibrate<br>Fenofibrate<br>Gemfibrozil<br>Benzafibrate<br>Ciprofibrate | Several renal/ hepatic disease<br>Diabetic nephopathy<br>Breast-feeding[2] |
| **Bile-acid sequestrants (resins)** | Plasma cholesterol reduction | Disruption of enterohepatic recycling<br>↑Stool elimination of bile acids<br>↓ Serum cholesterol | ↓ LDL (10-20%)<br>↓ HDL (1-2%)<br>↑TG (possible) | Gastrointestinal distress,<br>Constipation | Cholestyramine<br>Colestipol<br>Colesevelarm | Complete biliary obstruction |

[1] Including cytochrome P450 inhibithors, cyclosporine (an immunosuppressant drug) and several antifungal agents.
[2] Relative contraindication. Myopathy: muscular dysfunction; Hyperuricemia: abnormal elevated levels of uric acid in blood; Dyspepsia: impaired digestion. ↑: Increase; ↓: decrease; TG: triglyceride.

Statins are used to suppress cholesterol biosynthesis and improve the hepatic LDL clearance through the inhibition of HMGCR enzyme (Hebert et al., 1997; Krukemyer and Talbert, 1987). While bile acid sequestrants reduces serum cholesterol levels by promoting the elimination of bile acids, niacin induces HDL synthesis and represses the hepatic VLDL synthesis (Handelsman, 2010; Kamanna and Kashyap, 2008; Vasudevan and Jones, 2006). The regulation of cholesterol absorption through ezetimibe as well as the stimulation of fatty acid oxidation through fibrates, are also important goals in lipid homeostasis management (Enjoji et al., 2010; Garcia-Calvo et al., 2005; van Raalte et al., 2004)

As in T2DM, combination therapy is also inevitable in many cases to achieve lipid goals. Although, to avoid adverse drug reactions and drug-drug interactions, special considerations need to be taken into account before starting this kind of therapy. Gemfibrozil (fibrate)-statin combined therapy increases the risk of myophathy (muscular disease), than other fibrate-statin combinations (Prueksaritanont et al., 2002; Shek and Ferrill, 2001). In fact, when coadministrated with a statin, fenofibrate showed to be more effective in maintaining lipid homeostasis (Grundy et al., 2005; Koh et al., 2005). In addition, antioxidant supplements in combination therapy with simvastatin (statin) and nicotinic acid can block the HDL response to this drug regimen (Cheung et al., 2001).

The side effects and the cost-effectiveness of the current pharmacological interventions are worrying and elevated, which enhance the needed of more effective and less costly approaches than the available ones.

### 1.5.2. Medicinal plants: the genus *Salvia*

Since earliest times that plants have been used for a large range of purposes including medicine, nutrition, flavourings and cosmetics, and their beneficial properties have been well reported. Nowadays, there is a resurgent interest in herbal remedies for diabetes mellitus prevention.

Derived from the Latin *salvere* that means “to heal”, the genus *Salvia* (family Laminaceae) comprises several species which have been known for their medicinal properties (Dweck, 2000; Miura et al., 2002; Wang et al., 2000). The dialect “sage” is attributed to several aromatic plants of the genus *Salvia* that have been described to

possesses, among others, anti-inflammatory, antioxidant and hypoglycaemic properties (Lima et al., 2006b; Lima et al., 2007). In ancient times, *Salvia* species were used to treat various illnesses such as epilepsy, colds, haemorrhage and tuberculosis (Topcu, 2006). Therefore, sage plants' reputation gave rise to popular sayings like the one found in 11$^{th}$ and 12$^{th}$ centuries *"why should a man die, if sage grows in his garden?"* (Salerno Medical School) or like an English maxim *"He that would live for aye, must eat sage in May?"* (Dweck, 2000). About 900 species from the genus *Salvia* are identified and despite their Mediterranean nature, they are found as naturalized plants in numerous and distinct parts of the globe (Karousou R et al., 2000; Topcu, 2006). Several *Salvia* species have long been used by folk medicine to treat diabetes, including *Salvia officinalis, Salvia fruticosa, Salvia lavandulifolia* and *Salvia plebeia* (Alarcon-Aguilar et al., 2002).

The small and evergreen perennial *Salvia officinalis* L. (**Figure 12**) is also known as common sage, garden sage or Dalmatian sage. *S. officinalis* have been reported to possess beneficial effects on glycaemic control (Alarcon-Aguilar et al., 2002). Eidi and Eidi (2009) confirmed the antidiabetic effect of *S. officinalis* ethanolic extract in normal and streptozotocin-induced diabetic rats. According to these authors, the sage extract not only significantly decreased serum glucose, triglycerides and cholesterol levels, but also increased serum insulin levels in treated diabetic animals (Eidi and Eidi, 2009). Treatment of rats with common sage extract (prepared as a tea) had shown decreased hepatocytes' response to glucagon and increased insulin sensitivity of hepatocyte primary cultures (Lima et al., 2006a). Moreover, the same study revealed that 14 days treatment of mice with *S. officinalis* tea decreased fasting glucose levels without changing glucose clearance after an intraperitoneal glucose tolerance test (Lima et al., 2006a). A recent study showed that sage may be used to treat diabetic-associated lipid abnormalities, through improving lipid profile in patients with primary hyperlipidemia (Kianbakht et al., 2011).

*Salvia triloba* L. or Greek sage are both synonyms for *Salvia fruticosa* Mill. (**Figure 12**), a medicinal plant that contains leaves with three (tri) lobes (loba). The hypoglycaemic potential of this plant was demonstrated in both normoglycaemic and alloxan-hyperglycaemic rabbits. The authors concluded that this potential was due to a reduction of the intestinal glucose absorption, since no modifications on plasma insulin levels were detected (Perfumi et al., 1991). More recently, *S. fruticosa* tea demonstrated

to be effective in stabilising fasting blood glucose levels in streptozotocin-diabetic rats. This effect was accompanied with a decrease of SGLT1 levels in BBM (Azevedo et al., 2011).

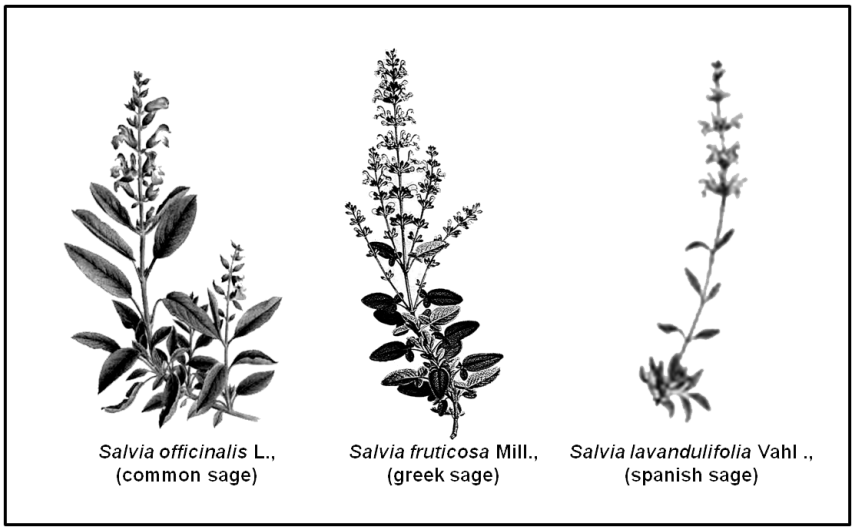


**Figure 12.** Some species of the genus *Salvia*.

*Salvia lavandulifolia* Vahl. (**Figure 12**) (Spanish sage) is among those that have been studied in normal and alloxan- or streptozotocin-diabetic animals, the plant to which the best results were observed (Alarcon-Aguilar et al., 2002; Zarzuelo et al., 1990). Zarzuelo et al. (1990) demonstrated that the hypoglycaemic potential of *S. lavandulifolia* extract results from a decrease on intestinal glucose uptake and to the increase of peripheral glucose uptake. *S. lavandulifolia* is, however, still poorly studied (Baricevic D and Bartol T, 2000; Zarzuelo et al., 1990).

The wide range of medicinal properties and the increasing number of scientific reports that confirm the health benefits of these sage species, highlight the advantage of considering them good candidates to be included in pharmaceutical and food industries. However, it is crucial to continue the efforts to elucidate the active principles and the mechanisms of action, which in long term may be accountable for the general health improving properties reported to sage species.

#### 1.5.2.1 The bioactive compounds of sage species

Since antiquity that *Salvia* species have been extensively used as pharmaceutical and therapeutical plants by folk medicine. This fact had rouse interest in investigating the medicinal properties of individual compounds that constitute extracts prepared from

aerial parts and/or roots of the herb (Panagiotopoulos E et al., 2000). Only the active compounds are worthy of investigation and designed as secondary metabolites since apparently, they are not involved directly in plant growth or development (Panagiotopoulos E et al., 2000).

The well acceptance by the consumers as well as the fewer reported side effects associated to normal dosages, makes the natural compounds reliable alternatives to synthetic drugs available in the market. However, only small amounts can be extracted from large amounts of the plant fraction and so, at the end of the process the product yield is very low. New strategies are being applied in order to face this disadvantage and potentiate massive production of plant secondary metabolites (Panagiotopoulos E et al., 2000). *Salvia* species contain a complex mixture of secondary metabolites, such as phenolic compounds, which can be grouped in two classes: phenolic acids and flavonoids (**Table 4**).

**Table 4.** Phytochemical characterization of the phenolic compounds (µg/ml) present in water extracts (WE) of some *Salvia* species. Adapted from [Azevedo, 2008; Lima et al., 2006b].

| Component | *S. officinalis* (µg/ml WE) | *S. fruticosa* (µg/ml WE) | *S. lavandulifolia* (µg/ml WE) |
|---|---|---|---|
| *Phenolic compounds* | | | |
| *Phenolic acids* | | | |
| rosmarinic acid | 518.5 | 577.3 | 146.4 |
| caffeic acid | 8.2 | 8.7 | - |
| ferulic acid | 5.2 | 3.5 | 6.5 |
| 3-caffeoylquinic acid | tr | tr | tr |
| 5-caffeoylquinic acid | tr | tr | tr |
| *Flavonoids* | | | |
| 6-hydroxyluteolin-7-glucoside | - | 104.8 | - |
| not identified flavone* | - | 99.1 | - |
| apigenin-7-glucoside | 4.3 | 6.7 | - |
| luteolin-7-glucoside | 196.3 | tr | 29.6 |
| 4',5,7,8-tetrahydroxyflavone | 9.0 | - | - |

Tr: Trace amounts (compounds present in concentration below than 0.1 µg/ml).
*Quantified as apigenin-7-glucoside.

Among the non-flavonoids, phenolic acids assume a relevant position. Rosmarinic acid (RA; **Figure 13**), originally identified in *Rosmarinus officinalis* L. (rosemary), is the major phenolic compound of *S. fruticosa*, *S. officinalis* and *S. lavandulifolia* aqueous extracts (**Table 4)**. A plethora of biological properties have been attributed to RA ranging from antibacterial and antiviral to antioxidant and anti-inflammatory (Huang and Zheng, 2006). RA showed promising evidences in preventing the progression of diabetic nephropathy through decreasing the glomerular hypertrophy and reducing lipid peroxidation in diabetic rats (Tavafi et al., 2010). The antioxidant properties of RA may explain the observed improvement in insulin sensitivity in fructose-fed mice (Vanithadevi and Anuradha, 2008). In addition, RA also inhibited porcine pancreatic α-amylase activity (McCue and Shetty, 2004). More recently, Azevedo and colleagues (2011) reported that 4 days treatment with RA decreased blood glucose levels and significantly inhibited the carbohydrate-stimulated adaptive increase of SGLT1 in rat BBM.

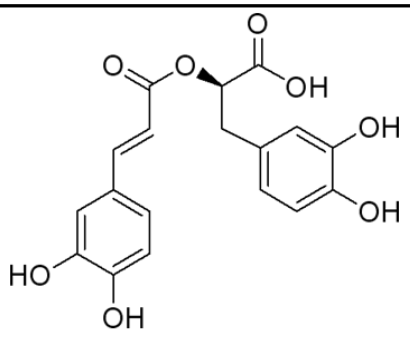


**Figure 13.** Structure of rosmarinic acid

Luteolin-7-glucoside (L7G, **Figure 14A**) is the most abundant flavonoid present in both *S. officinalis* and *S. lavandulifolia* extracts (**Table 4**). Although, *S. fruticosa* extracts contains only trace amounts of L7G (**Table 4**). Dietary supplementation with L7G showed to improve plasma glucose and decrease total and LDL cholesterol levels in healthy rats (Azevedo et al., 2010).

Dietary L7G is biotransformed in the intestine by microorganisms and hydrolases into its aglucone luteolin (3',4',5,7-tetrahydroxyflavone; **Figure 14B**). Luteolin has a variety of biological activities including anti-inflammatory, antimutagenic, and antitumorigenic properties (Jang et al., 2008; Ross and Kasum, 2002). In a study performed with 21 flavonoids, luteolin possessed the strongest inhibitory effect on α-glucosidase and α-amylase enzymes activities (Kim et al., 2000). In 3T3-L1 and mouse primary adipose cells, luteolin induced glucose uptake and Akt2 phosphorylation, under insulin stimulation and decreased gene expression of the inflammatory markers TNF-α and interleukin-6. The same study showed a luteolin-mediated induction of PPARγ expression (Ding et al., 2010).

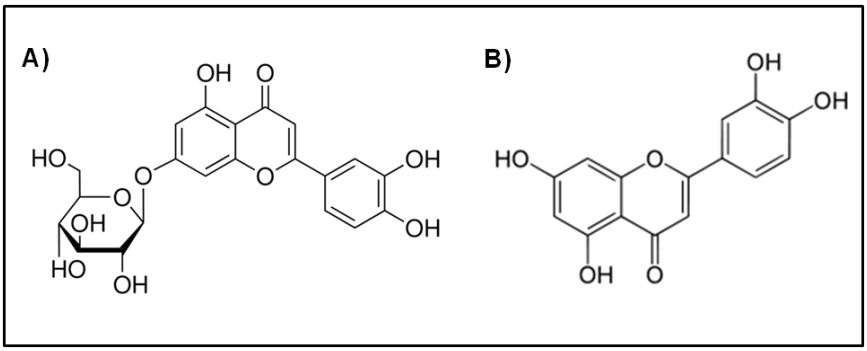


**Figure 14.** Structure of luteolin-7-glucoside (**A**) and luteolin (**B**).

The ability for luteolin to restrain atherosclerosis onset is based on several evidences ranging from the inhibition of cholesterol and fatty acid synthesis to prevention of LDL oxidation (Ashokkumar and Sudhandiran, 2008; Lee et al., 2010; Tuansulong et al., 2011). Finally the antioxidant activity of luteolin showed to protect against the development of diabetic nephropaty (Wang et al., 2011).

Therefore, some natural products seemed to be good candidates for being used as functional foods and/or food supplements. Nevertheless, in regard to health improving effects and mechanism of action, much yet remains to be investigated, which highlight the need of further studies.

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# CHAPTER 2

## *Sage tea drinking: a pilot trial*

## 2.1. Chapter overview

Among all the beneficial properties attributed to *Salvia officinalis*, its reported antidiabetic potential engaged our interest. The work presented in this chapter comprises the results from a pilot study in which the beneficial effects of *S. officinalis* water extract (prepared as a tea) were evaluated in healthy female volunteers that, according to their age, constitute a group of risk for developing T2DM.

Our results showed that drinking sage tea does not cause hepatotoxicity or other adverse effects such as changes in blood pressure, body weight and heart rate at rest, therefore it is safe. The risk of hypoglycaemia events associated with sage tea drinking was also excluded since no effects on fasting/postprandial blood glucose levels were detected. An improvement in lipid profile was also observed by increasing plasma HDL levels and gradually decreasing total and LDL cholesterol levels, which contributes to positively control dyslipidemia and prevent CVDs, strongly related with T2DM. Sage tea drinking showed to be valuable in improving the antioxidant status of the volunteers, since it increased the activity of the erythrocytes' antioxidant enzymes (SOD and CAT).

The observed results not only point out the acclaimed antidiabetic potential of *S. officinalis* tea but also denote/highlight the harmless nature of this plant.

## 2.2. Publication

This chapter comprises the following publication:

**Carla M. Sá**, Alice A. Ramos, Marisa F. Azevedo, Cristovao F. Lima, Manuel Fernandes-Ferreira and Cristina Pereira-Wilson (**2009**). Sage Tea Drinking Improves Lipid Profile and Antioxidant Defences in Humans. *Int J Mol Sci* **10**, 3937-3950.

# Sage Tea Drinking Improves Lipid Profile And Antioxidant Defences in Humans

Carla M. Sá[1], Alice A. Ramos[1], Marisa F. Azevedo[1], Cristovao F. Lima[2], Manuel Fernandes-Ferreira[2] and Cristina Pereira-Wilson[1]

[1] *CBMA–Centre of Molecular and Environmental Biology/Department of Biology, School of Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.*

[2] *CITAB–Centre for the Research and Technology of Agro-Environment and Biological Sciences/Department of Biology, School of Sciences, University of Minho, 4710-057 Braga, Portugal.*

## Abstract

*Salvia officinalis* (common sage) is a plant with antidiabetic properties. A pilot trial (non-randomized crossover trial) with six healthy female volunteers (aged 40-50) was designed to evaluate the beneficial properties of sage tea consumption on blood glucose regulation, lipid profile and transaminase activity in humans. Effects of sage consumption on erythrocytes' SOD and CAT activities and on Hsp70 expression in lymphocytes were also evaluated. Four weeks sage tea treatment had no effects on plasma glucose. An improvement in lipid profile was observed with lower plasma LDL cholesterol and total cholesterol levels as well as higher plasma HDL cholesterol levels during and two weeks after treatment. Sage tea also increased lymphocyte Hsp70 expression and erythrocyte SOD and CAT activities. No hepatotoxic effects or other adverse effects were observed.

**Keywords:** *Salvia officinalis* L.; type 2 diabetes mellitus; lipid profile; human trial; antioxidant defences

## 1. Introduction

Diabetes mellitus is a serious public health problem characterized by deficient plasma glucose regulation due to tissue insulin resistance and/or β-cell failure which causes high morbidity and mortality rates. Type 2 diabetes mellitus (T2DM) accounts for the majority cases of diabetes (about 90%) and is becoming more prevalent due to the increasing rates of obesity in youth and adulthood and sedentary lifestyles (Williams and Pickup, 2004).

Dyslipidemia is also common among diabetic patients and plays a critical role in the development of cardiovascular complications. Metabolic dyslipidemia is characterised by high levels of triglycerides, associated with low levels of high-density lipoprotein cholesterol–HDL-C with or without a raise in low-density lipoprotein cholesterol–LDL-C (Moller, 2001; Saxena et al., 2005; Toth, 2005; Veiraiah, 2005). These imbalances in the internal metabolic environment, combined with the characteristic low antioxidant defences of diabetics can lead to oxidative stress and cellular damage. Oxidative stress has been demonstrated to be a contributor to the progression of the disease, accelerating both β-cell failure and cardiovascular complications. Antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) play crucial roles in the cellular protection against oxidative damage eliminating reactive oxygen species (ROS) (Celik and Isik, 2008; Mates et al., 1999).

The increased expression of heat shock proteins (Hsp) is regarded as one of the most powerful means of cytoprotection against loss of cellular homeostasis and Hsp levels have been shown to be involved in tissue insulin responsiveness (Feder and Hofmann, 1999). The study of levels of cell protection in the most relevant insulin sensitive tissues is highly invasive and the easily accessible lymphocyte may provide valuable biomarkers of health status (Bonassi and Au, 2002; Jin et al., 2004). The Hsp70 levels of lymphocytes may therefore provide information on effects on insulin response.

T2DM is preventable through lifestyles changes (including diet changes, physical exercise and weight loss) and pharmacological interventions with drugs such as metformin and acarbose (Costacou and Mayer-Davis, 2003; Gruber et al., 2006; Jermendy, 2005). Herbal teas with glucose-lowering properties may offer low-cost alternatives to pharmacological interventions to limit the progression of the disease while having good acceptance. In particular *Momordica charantia* has been shown to improve insulin secretion in β-cells, increase peripheral glucose uptake, significantly reduce serum cholesterol and triglycerides levels at the same time as increasing HDL-C levels (Fernandes et al., 2007); *Coccinia indica* improves antioxidant status by increasing antioxidant defences such as SOD, CAT and reduced glutathione levels and shows a significant hypoglycaemic action by decreasing blood glucose levels and increasing hepatic glycogen synthesis in animal models (Kumar et al., 1993; Venkateswaran and Pari, 2003) and *Camellia sinensis*, has been associated with weight reduction, decrease in blood pressure and blood glucose levels, protection against lipid

peroxidation and improvement of blood lipid profile which suggest beneficial effects against obesity, cardiovascular diseases (CVDs) and T2DM (Coimbra et al., 2006a; Coimbra et al., 2006b; Polychronopoulos et al., 2008).

Common sage (*Salvia officinalis*) is among the plants to which antidiabetic properties have been attributed by popular medicine and its extracts showed to possess hypoglycaemic effects in normal and diabetic animals (Alarcon-Aguilar et al., 2002; Eidi et al., 2005). In a previous study we have shown that treatment with sage tea for 14 days lowered fasting plasma glucose levels but had no effects on glucose clearance in response to an intraperitoneal glucose tolerance test (ipGTT) in rats (Lima et al., 2006a). Using hepatocyte primary cultures a decreased gluconeogenesic response to glucagon and a higher responsiveness to insulin were found after *in vivo* treatment with sage tea (Lima et al., 2006a). *In vivo* treatments with *Salvia fruticosa* tea also reduced plasma glucose in streptozotocin rats (unpublished observations).

With the purpose of studying the effects of sage tea consumption on glucose regulation in humans, a pilot trial with human volunteers was carried out where a number of parameters relevant to diabetes were analysed such as fasting and postprandial blood glucose, response to an oral glucose tolerance test–OGTT, lipid profile, liver toxicity and antioxidant defences. Demonstration that there is no toxicity or adverse effects associated with sage consumption paves the way for future studies involving diabetic patients where the true antidiabetic potential of sage will have to be tested.

## 2. Experimental Section

### *2.1. Subjects and study design*

Six healthy female volunteers (aged 40-50) participated in this trial after signing an informed consent form. The whole study was carried out in accordance with the principles of the Declaration of Helsinki. Smokers and subjects on regular medication were excluded from the study. Effects of sage tea drinking on body weight, blood pressure and heart rate at rest were recorded at first week of baseline and the end of each of the eight weeks of the trial. Weekly records of perceived negative events and concomitant medication were also kept. All the volunteers completed the study and reported no side effects. A non-randomized crossover study, where individuals serve as

their own controls, was carried out in three phases: two weeks of baseline, four weeks of sage tea treatment and two weeks of wash-out (**Figure 1**). The two-week baseline phase was included in order to obtain control values for all the volunteers. During this phase, all the parameters were measured and values are presented in figures and tables as basal levels. A treatment phase with sage tea followed, where 300 ml of tea were taken twice daily for four weeks. Sampling was carried out at the end of second and fourth week of sage treatment. A two-week wash-out phase was included after treatment with the aim to assess the duration of sage tea effects beyond the treatment period.


**Figure 1.** Experimental outline of the pilot study. Blood samples were taken at the times indicated by the arrows. Oral glucose tolerance test were performed at the times indicated by the circles.

*2.2. Plant material and preparation of S. officinalis tea*

*Salvia officinalis L.* plants were grown in an experimental farm located in Arouca, Portugal, and were collected in April, 2001. The aerial parts of plants were lyophilized and kept at -20°C. The sage tea was routinely prepared by pouring 300 ml of boiling water onto 4 g dried plant material and allowing to steep for 5 min (Lima et al., 2005). This infusion yielded about 3.5 ± 0.1 mg lyophilized extract dry weight per ml, where rosmarinic acid (362 mg/ml infusion) and luteolin-7-glucoside (115.3 mg/ml infusion) were the major phenolic compounds, and 1,8-cineole, *cis*-thujone, *trans*-thujone, camphor and borneol the major volatile components (4.8 mg/ml infusion). For full extract characterization see (Lima et al., 2005).

*2.3 Blood samples, erythrocytes' hemolysates and lymphocytes lysates*

At the different sampling points (baseline–B, second week of treatment–T2, fourth week of treatment–T4 and at the end of wash-out–W), venous blood samples were collected postprandially in EDTA vacutainers (Vacuett®, Greiner Bio-one GmbH, Austria). An aliquot of blood was used for measuring glucose levels. Immediately after sampling, about 3 ml of blood were centrifuged at 200 × g (KUBOTA 2100, Tokyo, Japan) for 10 min to separate the plasma. Plasma aliquots were stored at -80°C for later

measurements of transaminases, total cholesterol, HDL-C and LDL-C levels. The remaining erythrocyte enriched fraction was haemolysed to analyse SOD and CAT activity. About 10 ml of blood were used to separate peripheral blood lymphocytes (PBLs) by a Ficoll density gradient centrifugation following the procedure provided by the Ficoll manufacturer (Ficoll Paque- Plus, GE Healthcare, Piscataway, NJ, USA). The resultant PBL fraction was collected, washed with PBS and the cell pellet was homogenised with lysis buffer (25 mM $KH_2PO_4$, pH 7.5, 2 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA, 1 mM EGTA, with 0.1 mM PMSF and 2 mM DTT added fresh). Protein concentration from lymphocyte lysates was measured with the Bradford reagent (Sigma-Aldrich, Inc., St. Louis, MO, USA) and aliquots kept at -80°C for later quantification of Hsp70.

### *2.4. Measurement of blood and plasma parameters*

#### 3.4.1. Quantification of glucose levels and oral glucose tolerance test (OGTT)

Fasting and postprandial glycaemia were measured with the Accutrend® GCT device (Roche Diagnostics GmbH, Mannheim, Germany) using Accutrend® test strips for glucose (Roche Diagnostics GmbH). Two OGTTs were performed after an overnight fast one at baseline and the other at week four of sage tea treatment. For that, 1 g of glucose per kg body weight of each volunteer was given in up to 300 ml of warm water, which was consumed within 5 min of start. The OGTT started when the subjects began drinking with blood sampling taken before as well as at 45 min and 165 min after the oral glucose load. Blood glucose concentration was measured as above.

#### 2.4.2. Characterization of lipid profile

Total plasma cholesterol, LDL-C and HDL-C levels were measured in plasma using spectrophotometric commercial kits from Spinreact (Girona, Spain), according to the manufacturer's specifications.

#### 2.4.3. Quantification of plasma aminotransferases

The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured spectrophotometrically in plasma following the NADH

oxidation method at 340 nm on a plate reader (Spectra Max 340 pc, Molecular Devices, Sunnyvale, CA, USA), as previously described (Lima et al., 2005).

2.4.4. Quantification of erythrocytes' antioxidant defences

The haemolysate fraction was used to determine SOD activity using the Ransod kit (Randox, Crumlin, UK) following the manufacturer's specifications. The SOD activity in haemolysates was expressed as U/ml, with 1U corresponding to 50% of inhibition of 2-(4-iodophenyl)-3-(4-nitro-phenol)-5-phenyltetrazolium chloride (INT) reduction under assay conditions. The same haemolysates were used to measure CAT activity as described elsewhere (Aebi, 1984). In brief, the decomposition of $H_2O_2$ was followed at 240 nm in a spectrophotometer (Cary IE, UV-Visible Spectrophotometer Varian, Australia) and the activity expressed as U/ml (U being μmol of $H_2O_2$ decomposed per minute) using the molar extinction coefficient of 0.0394 ml $\mu mol^{-1}$ $cm^{-1}$.

2.4.5. Western blot analyses

The quantification of Hsp70 protein in lymphocyte lysates was assessed by Western Blot in which proteins (20 μg per sample) were separated by SDS-PAGE using the mini-PROTEAN 3 electrophoresis cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins were then transferred onto Hybond-P polyvinylidene difluoride membrane (GE Healthcare, UK) and membranes blocked in 5% (w/v) non-fat dry milk in TPBS (0.05% (v/v) Tween 20 in PBS, pH 7.4). Blotted membranes were probed with mouse monoclonal antibodies against Hsp72 (StressGen, Assay Designs, Inc., Ann Arbor, MI, USA) and β-Actin (Sigma-Aldrich; used as loading control). Bound antibodies were then detected by chemiluminescence using appropriate secondary antibodies and the reactive bands acquired with a ChemiDoc XRS (Bio-Rad) imaging densitometer. Band intensity was quantified using the Quantity One image analysis software (Bio-Rad).

*2.5. Statistical analysis*

Data are expressed as means ± SEM (n=6). For statistical analysis the different parameters were analysed by repeated one-way ANOVA measurements followed by the Student-Newman-Keuls post-test (GraphPad Prism, version 4.03; GraphPad

Software Inc., San Diego, CA, USA) to identify differences between studied time points. *P* values ≤ 0.05 were considered statistically significant (with a confidence interval of 95%).

## 3. Results and Discussion

### *3.1 Effects of Salvia officinalis on blood glucose regulation, plasma aminotransferase activity, blood pressure, heart rate at rest and body weight*

In this study we evaluated in healthy women volunteers the effects of *Salvia officinalis* (sage) tea drinking (300 ml, twice a day) on parameters relevant to diabetes and its associated cardiovascular complications. In spite of its claimed antidiabetic potential and traditional use, no effects on blood glucose were observed in healthy humans (**Table 1**). In our previous work, sage tea drinking decreased fasting blood glucose in normoglycaemic mice (Lima et al., 2006a). Since no such effects on fasting blood glucose were found in the present study (**Table 1**), the risk of hypoglycaemia is excluded. Sage tea drinking improved lipid profile and increased antioxidant defences (see below) which may indirectly improve the diabetic condition.

**Table 1.** Physiological and biochemical parameters during the different phases of the trial: baseline (B), second (T2) and fourth (T4) week of sage tea treatment and wash-out (W).

| Parameters | Phases of the trial | | | |
|---|---|---|---|---|
| | B | T2 | T4 | W |
| Weight (kg) | 56.2 ± 7.1 | 56.2 ± 6.1 | 55.6 ± 6.1 | 55.9 ± 5.9 |
| Systolic blood pressure (mmHg) | 116.1 ± 10.3 | 110.3 ± 14.5 | 110.7 ± 15.5 | 107.7 ± 13.2 |
| Diastolic blood pressure (mmHg) | 68.2 ± 9.4 | 64.5 ± 14.0 | 63.6 ± 11.7 | 59.5 ± 9.1 |
| Heart rate at rest (beats/min) | 65.9 ± 10.7 | 67.2 ± 10.9 | 66.6 ± 8.2 | 68.7 ± 9.9 |
| ALT (IU/l) | 7.3 ± 1.0 | 6.8 ± 1.4 | 8.4 ± 1.6 | 7.6 ± 1.5 |
| AST (IU/l) | 8.1 ± 1.1 | 10.0 ± 2.0 | 10.6 ± 1.8 * | 9.8 ± 1.2 |
| Fasting glucose levels [a] (mM) | 4.31 ± 1.18 | 4.60 ± 0.92 | 4.21 ± 1.54 | --- |
| Postprandial glucose levels [a] (mM) | 5.33 ± 1.64 | 4.35 ± 0.53 | 4.88 ± 0.94 | 4.58 ± 0.90 |

Values are mean ± SEM (n=6). *$P \leq 0.05$ when compared with baseline values. [a] Glucose concentration in blood.

Plasma aminotransferase AST and ALT activities were determined in order to evaluate the safety of *S. officinalis* tea drinking in humans. Although a significant increase in plasma AST enzyme activity was observed at the fourth week of sage tea treatment (**Table 1**), toxicity did not occur, since the results are well below reference values (40 IU/l) (Jamal et al., 1999; Kim et al., 2004). Thus, drinking sage tea does not cause hepatotoxicity nor does it induce other adverse effects, such as changes in blood pressure, heart rate at rest and body weight (**Table 1**).

In order to assess the effects of sage tea on glucose clearance, two OGTTs were performed, at baseline and at the end of sage tea treatment (four weeks after the first one), and no changes were observed (**Table 2**). Although all the volunteers were non-diabetic, they belong to an age group at risk of developing impaired glucose tolerance (IGT) (a pre-diabetic stage). All subjects showed no glucose intolerance. Although no effects on glucose regulation were observed in healthy humans, it remains to be established whether sage tea drinking helps to regulate blood glucose in hyperglycaemic patients.

**Table 2.** Blood glucose concentration in response to an oral glucose tolerance test (OGTT).

| | **Blood glucose levels (mM)** | | |
|---|---|---|---|
| **Time** | **0 min** | **45 min** | **165 min** |
| **Baseline** | 4.3 ± 1.2 | 5.5 ± 1.2 | 4.3 ± 0.4 |
| **Treatment (4 weeks)** | 4.2 ± 1.5 | 6.9 ± 1.4 | 4.5 ± 0.9 |

Values are mean ± SEM (n=6). The reference values for a non-diabetic individual to the standard OGTT (75 g glucose/300 ml water) are: 3.33–5.56 mM (before glucose loading); < 10 mM (0.5–1.5 h after glucose loading) and 3.33–5.56 mM (3 h after glucose loading) (Ravel, 1989).

### *3.2. Effects of Salvia officinalis on lipid profile*

Sage tea treatment reduced slightly plasma total cholesterol levels during treatment phase (by 4.5% at T2 and by 5.3% at T4), achieving a significant reduction two weeks after the end of the treatment (values 16% lower than the baseline; **Figure 2A**). A beneficial effect on lipoprotein levels, with a gradual reduction of LDL-C (of 12.4% at the end of the treatment and 19.6% after 2 weeks wash-out; **Figure 2C**) and a gradual increase of HDL-C levels (50.6% at the end of the treatment and 37.6% after two weeks wash-out; **Figure 2B**) were observed. The LDL-C/HDL-C ratio contributes to assess the risk of cardiovascular complications due to dyslipidemia (Sullivan, 2002). As shown in **Figure 2D**, sage tea gradually decreased LDL-C/HDL-C ratio from

baseline until the end of four weeks of tea treatment. This ratio remained significantly reduced even after the two week wash-out period (**Figure 2D**). These results suggest that *S. officinalis* tea consumption is accountable for the improvement of the lipid profile inducing a decrease on the highly atherogenic LDL-C particles (which are easily oxidable and less readily cleared (Nesto, 2005) and an increase in the HDL-C particles, contributing, therefore, positively to the control of the dyslipidemia frequently observed in T2DM but also related to other diseases.

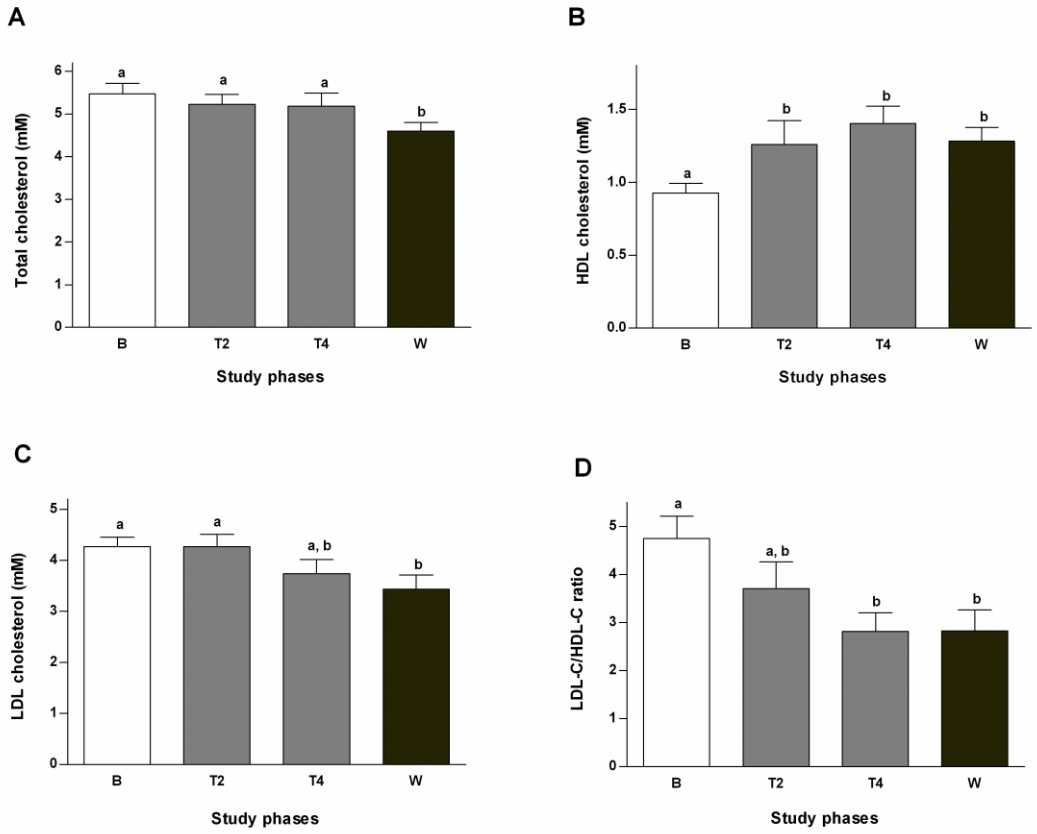


**Figure 2.** Total cholesterol [**A**], LDL cholesterol [**B**] and HDL cholesterol [**C**] levels as well as LDL-C/HDL-C ratio [**D**] in plasma measured at different points during the study: baseline (B), second (T2) and fourth week of treatment (T4), and wash-out (W). Values are mean ± SEM (n=6). Groups with the same letter notation are not significantly different from each other ($P > 0.05$).

A variety of pharmaceutical approaches have been developed in order to achieve both decrease of LDL-C and rise of HDL-C levels, with the aim to reduce the risk of CVDs (Toth, 2005). Despite the available therapies based on statins, niacin and fibrates (pharmacological agents used to lower plasma LDL-C and increase HDL-C levels), the need for more effective drugs drives the search for alternative compounds. Several natural compounds have been shown to act on cholesterol metabolism (by reducing its

absorption or its synthesis), such as phytosterols and catechins (Plana et al., 2008; Raederstorff et al., 2003). Extracts from some sage species have been shown to be effective in the prevention of cardiovascular diseases due to, at least in part, prevention of LDL-C oxidation (Chen et al., 2001). Sage tea drinking had no significant effects on post-prandial triglycerides (data not shown).

### *3.3. Effects of Salvia officinalis on antioxidant defences and heat-shock protein 70 (Hsp70) expression*

Sage tea drinking improved human erythrocyte antioxidant status by significantly increasing SOD and CAT activities after two weeks of sage treatment, returning afterwards to normal values (**Figure 3A and 3B**). The antioxidant properties of sage tea, in addition to preventing lipoprotein oxidation, may also protect cells from diabetes' related gluco- and lipotoxicity and prevent progressive β-cell destruction, which could provide long term protection of these insulin-producing cells.

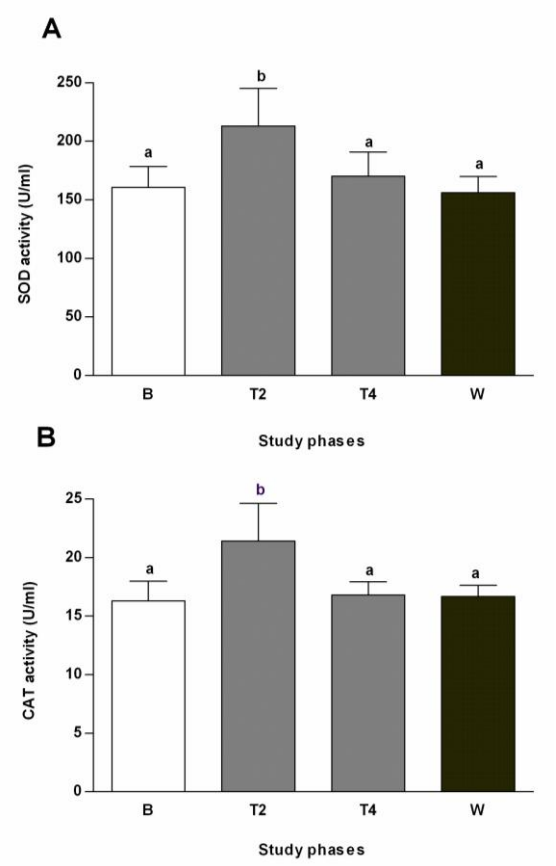


**Figure 3.** Antioxidant activities of SOD [**A**] and CAT [**B**] measured in haemolysed erythrocytes. Samples were taken at different time points during the study: baseline (B), second (T2) and fourth week of treatment (T4) and wash-out (W). Values are mean ± SEM (n=6). Groups with the same letter notation are not significantly different from each other ($P > 0.05$).

The antioxidant activity of phenolic compounds has been widely studied and it is known that these compounds can either stimulate endogenous antioxidant defence systems or scavenge reactive species (Scalbert et al., 2005). Rosmarinic acid and luteolin-7-glucoside are the two most representative phenolic compounds present in our *S. officinalis* extracts (tea) (Lima et al., 2005). These phenolic compounds showed protective effects against oxidative damage in hepatocytes, and limited GSH depletion induced by *tert*-butyl hydroperoxyde in HepG2, preserving cell viability (Lima et al., 2006b). The same happened for sage extracts in HepG2 cells (Lima et al., 2007b). In animals, sage tea drinking also stimulated several antioxidant enzymes in the liver (Lima et al., 2005; Lima et al., 2007a), corroborating the effects of this tea in human erythrocytes observed in the present study

Since lymphocytes may provide valuable and easily accessible biomarkers of the health status of individuals (Bonassi and Au, 2002; Jin et al., 2004) and heat shock proteins have been involved in tissue insulin responsiveness (Feder and Hofmann, 1999), the expression of Hsp70 in human lymphocyte lysates was evaluated. The lymphocyte's inducible Hsp72 protein not only significantly increased at the second week of *S. officinalis* tea treatment (about 2.8-fold) but also remained elevated in the wash-out period (**Figure 4**). These findings suggest a beneficial potential of sage tea drinking on Hsp72 protein induction, an endogenous stress modulator, which plays a crucial role in cellular homeostasis decreasing the risk of development of T2DM by blocking inflammatory signalling molecules including c-Jun N-terminal kinase (JNK), inhibitor of kB kinase (IKK) and tumor necrosis factor-α (TNF-α) in insulin responsive tissues (Gabai et al., 1997; McCarty, 2006; Meldrum et al., 2003; Park et al., 2001). These molecules phosphorylate insulin receptor substrate-1 (IRS-1) in specific serine sites and determine decreased insulin sensitivity. Indeed, Hsp72 gene and protein expression has been shown to be significantly reduced in T2DM patients and correlated with reduced insulin sensitivity (Bruce et al., 2003; Chung et al., 2008; Kurucz et al., 2002). The antioxidant alpha-lipoic acid showed recently to improve insulin action in high-fat-fed rats by increasing the expression of Hsp72 and consequently inhibiting JNK and IKK (Gupte et al., 2009). Therefore, an increase in inducible Hsp70 protein expression by sage tea would represent an amelioration of whole-body insulin sensitivity although the assumption that lymphocyte Hsp levels mimic other tissues Hsp levels requires further demonstration.

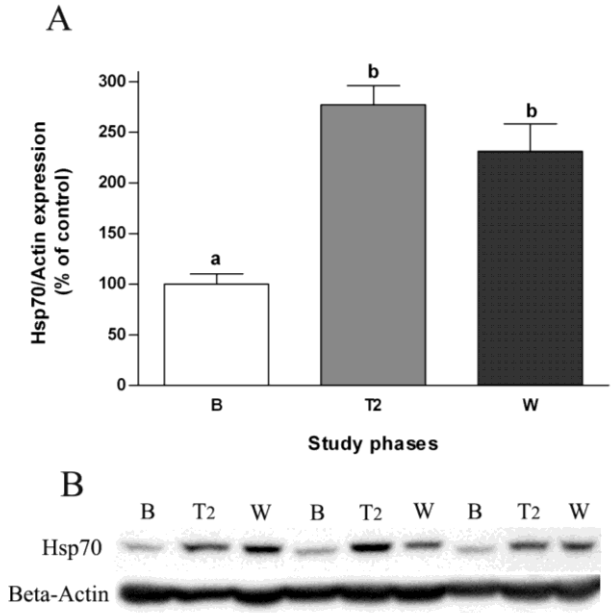


**Figure 4.** Western blot analysis of the inducible form of Hsp70 (Hsp72) in human lymphocytes at the different points during the study: baseline (B), second week of treatment (T2) and wash-out (W). [**A**] Values are mean ± SEM (n=6). Groups with the same letter notation are not significantly different from each other ($P > 0.05$). [**B**] Representative immunoblots of three subjects (β-Actin was used as loading control).

Heat shock proteins confer also cytoprotection and assure survival after environmental stresses, being therefore implicated in infection, immunity and aging, as well as in ischemic and neurodegenerative diseases (Putics et al., 2008). Thus, induction of Hsp72 by sage tea could also be useful by conferring stress tolerance and cytoprotection against several environmental-induced injury conditions helping increase lifespan and prevent age-related diseases such as diabetes, cancer and neurodegeneration. Natural compounds such as resveratrol have been shown recently to induce the heat-shock response and to protect human cells from severe heat stress (Putics et al., 2008). As well, paeoniflorin isolated from *Paeonia lactifora* showed to induce heat shock proteins expression and to afford termotolerance in cultured cells (Yan et al., 2004).

## 4. Conclusions

In conclusion, a four week treatment with sage tea was effective in the improvement of lipid profile, antioxidant defences and lymphocyte Hsp70 protein expression of human volunteers, which in the long term may be responsible for the general health improving properties attributed to sage. Our results support the popular believe that *S. officinalis* tea is beneficial and although not demonstrating effects on glucose regulation in healthy individuals, they show that sage tea drinking is safe and pave the way for sage's effects to be tested in diabetic patients.

## Acknowledgements

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# CHAPTER 3

## *The role of phytochemicals in lipid metabolism under dietary manipulation*

## 3.1. Chapter overview

It is undeniable that lipids are crucial molecules in many biological processes and that disturbances in lipid homeostasis leads to dyslipidemia, a feature of the MetS and an underlying contributor to disease progression such as T2DM and CVDs. In this chapter, we reported the effects of *Salvia fruticosa* (SFT) and RA aqueous extracts on hepatic lipid metabolism, intestinal cholesterol absorption as well as on lipid profile in response to changes in dietary carbohydrate levels.

In this study, rats were fed with a low carbohydrate (Lc) diet for 7 days and then returned to a normal (referred as high carbohydrate, [Hc]) diet but only for 4 days (different groups drinking water or SFT or RA). Plasma total, LDL and HDL cholesterol levels were reduced in animals fed the Lc diet for 7 days. SFT treatment during the 4 days of Hc diet reintroduction, showed to increase plasma HDL levels although, neither SFT nor RA modified plasma total and LDL cholesterol levels. SFT and particularly RA prevented the return of plasma insulin to control levels, an effect that was accompanied by a decrease on the hepatic FAS protein expression. These effects on fatty acid synthesis seemed to be distinctly regulated: while RA showed to downregulate SREBP-1 gene expression, SFT showed to regulate the transcriptional activation of ChREBP. A significant inhibition of the HMGCR gene expression was mediated by both SFT and RA treatments. RA increased the mRNA levels of PPARα and its target gene CPT1, suggesting a potential role of this phenolic acid in stimulating fatty acid oxidation in the liver. Interesting was the finding that RA prevented the carbohydrate-associated raise of intestinal cholesterol absorption through decreasing the protein levels of BBM NPC1L1 transporter. The previous demonstration that both SFT and RA reduced the diet-induced boost of SGLT1 expression at the rat intestinal BBM[1], might point out an effect of these natural products on glucose and lipid metabolism.

Although further work must be conducted, the evidences here reported demonstrate that both SFT and RA may represent promising alternatives for preventing or delaying disease progression.

[1] Azevedo MF, Lima CF, Fernandes-Ferreira M, Almeida MJ, Wilson JM and Pereira-Wilson C (2011) Rosmarinic acid, major phenolic constituent of Greek sage herbal tea, modulates rat intestinal SGLT1 levels with effects on blood glucose. *Mol Nutr Food Res* **55 Suppl 1**:S15-25.

## 3.2. Manuscript 1

This chapter comprises the following manuscript:

**Carla M Sá**, Marisa Azevedo, Manuel Fernandes-Ferreira and Cristina Pereira-Wilson (**2012**). Sage tea drinking increases plasma HDL levels while its main phenolic rosmarinic acid stimulates PPARα, both affecting hepatic lipid metabolism (*in preparation*).

# Sage tea drinking increases plasma HDL levels while its main phenolic rosmarinic acid stimulates PPARα, both affecting hepatic lipid metabolism

Carla M. Sá[1], Marisa Azevedo[1], Manuel Fernandes Ferreira[2] and Cristina Pereira-Wilson[1]

[1] *CBMA - Centre of Molecular and Environmental Biology/ Department of Biology, University of Minho,4710-057 Braga, Portugal.*

[2] *CITAB - Centre for the Research and Technology of Agro-Environment and Biological Sciences/Department of Biology, Faculty of Sciences, University of Porto, Porto, Portugal*.

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## Abstract

We have recently confirmed that *Salvia officinalis* (family Laminaceae) water extract improves the lipid profile of normolipidemic subjects, suggesting the potential of this plant in preventing cardiovascular diseases (CVDs) progression, highly associated with type 2 diabetes mellitus (T2DM), and a feature of the metabolic syndrome (MetS). In addition, aqueous extracts of *Salvia fruticosa* (SFT) and particularly rosmarinic acid (RA), the main phenolic compound of both *Salvia* extracts, repressed the carbohydrate-induced increase of intestinal sodium-glucose cotransporter 1 (SGLT1). Since dietary carbohydrates are known to affect lipid metabolism, the present work aims to characterize the *in vivo* effects of SFT and RA in a number of parameters relevant to lipid metabolism, under dietary carbohydrate manipulation. Plasma HDL levels were improved in animals drinking SFT however, neither SFT nor RA drinking modified plasma total and LDL cholesterol levels. Both treatments significantly prevented the return of plasma insulin to control levels, and lead to a decrease in fatty acid synthase (FAS) protein in liver homogenates apparently by two distinct pathways: while SFT led to a downregulation of carbohydrate responsive element-binding protein (ChREBP) transcription, RA downregulated the sterol regulatory element-binding protein-1 (SREBP-1) mRNA levels. Moreover, both SFT and RA treatments decreased the HMG CoA reductase (HMGCR) mRNA levels in the liver, while RA-stimulated the mRNA levels of peroxisome proliferator-activated receptor alpha (PPARα) and its target gene carnitine palmitoyltransferase 1 (CPT1), suggesting that this phytochemical may act as a PPARα agonist that promotes fatty acid oxidation. Also interesting was the

finding that the carbohydrate-induced raise of intestinal cholesterol uptake was prevented by RA via downregulation of Niemann-Pick C1-Like 1 (NPC1L1) in brush-border membrane (BBM). Our study shows that SFT and RA are promising agents for regulating lipid metabolism in response to dietary carbohydrates and may contribute to maintaining lipid homeostasis and prevent MetS and related disease progression.

**Key words:** *Salvia fruticosa* – rosmarinic acid – lipid profile – PPARα - NPC1L1

## 1. Introduction

*De novo* lipogenesis links carbohydrate and lipid metabolism, the two energy reserves for the human body (Shiota and Magnuson, 2008). Any disruption in those metabolic processes results in serious changes in carbohydrate and lipid metabolism that may ultimately lead to disease development. The metabolic syndrome (MetS) is a cluster of metabolic abnormalities characterized by the coexistence of insulin resistance, visceral obesity, hyperglycaemia, hypertension and dyslipidemia (Laakso, 2001, Alberti and Zimmet, 2006, Mazzone et al., 2008). Visceral obesity and insulin resistance have been considered the major underlying contributors to MetS progression and this syndrome is associated with increased risk of developing cardiovascular diseases (CVDs) and type 2 diabetes mellitus (T2DM) (Balkau et al., 2007, Fulop et al., 2006).

High-fat diets are well-known to promote insulin resistance, increase body weight and body fat, hepatic steatosis and also impair repression of hepatic gluconeogenesis in rodent animals, while humans administrated with hypocaloric, low-fat diets showed to reduce lipid content in the liver (Petersen et al., 2005, Buettner et al., 2006, Marra et al., 2008). In addition, general increases in the consumption of carbohydrates are also correlated with metabolic disturbances leading to hyperlipidemia and hypertension in rodents (Hwang et al., 1987, Kasim-Karakas et al., 1996). When delivered to the liver in large quantities, glucose can be converted into fatty acids, which are then stored as triglycerides within the hepatocytes and/or exported into the blood as very low-density lipoprotein (VLDL). Thus, increased fat depots in the liver are highly related with increased serum low-density lipoprotein (LDL) and triglycerides, in combination with decreased high-density lipoprotein (HDL).

Dietary carbohydrates are also involved on the regulation of multiple lipogenic genes through the activation of transcription factors such as the carbohydrate responsive element-binding protein (ChREBP), a key moderator of glucose action in the regulation of lipogenic genes. In fact, inhibition of the hepatic ChREBP improved insulin resistance in ob/ob mice (Dentin et al., 2006, Iizuka et al., 2006) and prevented the transcriptional activation of glycolitic enzymes, such as Liver-type pyruvate kinase (L-PK) and lipogenic enzymes, like acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Dentin et al., 2004, Connor and Connor, 1997, Katan et al., 1997).

Additionally, the absorption of dietary carbohydrates also leads to changes in the pancreatic hormone insulin, which is known to stimulate lipogenesis (lipid synthesis) through a pathway that involve members of the family of transcription factors called the sterol regulatory element binding proteins (SREBPs), namely the SREBP-1c (Foretz et al., 1999, Azzout-Marniche et al., 2000, Shimomura et al., 1999b, Osborne, 2000). To date, three SREBP isoforms have been identified: SREBP-1a (more commonly found in cultured cells) and SREBP-1c, both protein products from a single gene, and SREBP-2. SREBPs isoforms are regulated by nutritional signals and intracellular cholesterol content. Under low-sterol levels, SREBP precursor is escorted from the endoplasmic reticulum (ER), where they are retained after translation, to the Golgi apparatus to be activated by proteolitic cleavage. Afterwards, the mature SREBP migrates to the nucleus where it targets the transcription of lipogenic and cholesterolgenic genes (Anderson, 2003, Rawson, 2003). While SREBP-1a isoform seems to be implicated in both lipogenic and cholesterogenic pathways (Eberle et al., 2004, Inoue et al., 2005, Amemiya-Kudo et al., 2002), SREBP-1c controls the transcriptional expression of genes involved in *de novo* fatty acid synthesis (like ACC and FAS) (Shimomura et al., 1997, Horton et al., 2002, Eberle et al., 2004). In contrast, SREBP-2 is a major regulator of the transcription of genes required for cholesterol uptake and synthesis (such as the 3-hydroxy-3-methylglutaryl coenzyme A reductase [HMGCR] and the low-density lipoprotein receptor [LDLR]) (Wong et al., 2006). HMGCR is responsible for converting HMG CoA into mevalonate, the rate-limiting step in the hepatic and intestinal *de novo* cholesterol pathway (Endo, 1992). Hepatic cholesterol synthesis is controlled by the quantity of dietary cholesterol that enters the liver, through hepatic surface receptors (like LDLR), via the chylomicron remnant pathway (Dietschy et al., 1993). Hence, alterations in intestinal cholesterol metabolism will be reflected in the hepatic cholesterol synthesis. SREBP-2 is also a regulator of the intestinal Niemann-

Pick C1-Like 1 (NPC1L1) cholesterol transporter, the molecular target of the intestinal cholesterol absorption inhibitor ezetimibe, that plays a crucial role in the maintenance of cholesterol homeostasis (Turley, 2008, Pramfalk et al., 2010). Through inhibiting the activity of intestinal NPC1L1, ezetimibe decreases sterol uptake and depresses plasma LDL cholesterol levels (Altmann et al., 2004, Davis et al., 2004, Goldberg et al., 2006). Thus, knowledge of whether lipid metabolism can be modified by diet composition, namely by carbohydrates, becomes pivotal to better understand the molecular mechanisms behind metabolic disorders and find new and more reliable therapeutical approaches.

In recent decades we have seen a renewed interest in traditional plant as new complementary and natural types of medicine, for the prevention and treatment of several diseases. Traditional medicine has empirically identified plants with lipid-lowering properties that provide good source material for the search of novel active compounds. We have recently reported that *Salvia officinallis* extract (prepared as a tea, [SOT]) consumption improves the lipid profile of normolipidemic subjects, by promoting a decrease in plasma total and LDL cholesterol and an increase in HDL cholesterol (Sa et al., 2009). These findings were recently corroborated in a randomized double-blind placebo-controlled clinical trial (Kianbakht et al., 2011). Although several reports documented the anti-inflammatory (El-Sayed et al., 2006, Kaileh et al., 2007), antioxidant (Exarchou et al., 2002) and hypoglycaemic (Karousou R et al., 2000) properties of *Salvia fruticosa* (Greek sage), its effects on lipid metabolism remains to be elucidated. The main phenolic compound of *S. officinalis* and *S. fruticosa* (sage) plants is rosmarinic acid, RA (contributing to 70% and 72% of all phenolic compounds present in both sages, respectively), which is also a common constituent of culinary herbs such as rosemary (*Rosmarinus officinalis* L.) (Al-Sereiti et al., 1999) and mint (*Mentha arvense* L.) (Ellis and Towers, 1970). A recent finding showed that both *S. fruticosa* (SFT) and RA aqueous extracts may modulate the trafficking of the intestinal sodium-glucose cotransporter 1 (SGLT1) and contribute to the control of plasma glucose levels (Azevedo et al., 2011). These evidences conduced us to the study present herein, where the effects of both SFT and RA drinking on several mediators of the lipid metabolism were evaluated, in response to dietary carbohydrates.

## 2. Materials and Methods

### *2.1 Reagents and antibodies*

Rosmarinic acid, anti-β-Actin antibody, Bradford reagent, HMGCR assay kit, Tri Reagent (Trizol eq.) as well as all other reagents were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. Commercial kits to quantify total cholesterol, LDL and HDL cholesterol and triglycerides were purchased from Spinreact (Girona, Spain). The commercial Rat Insulin ELISA Kit was acquired from Shibayagi Co., Ltd, (Gunma, Japan). Antibodies against phospho-ACC, total ACC, FAS and phospho-AMPK were purchased from Cell signalling (Danvers, MA, USA). The anti-AMPK total, anti-NPC1L1 and anti-PEPCK antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody against PCNA was obtained from Abcam (Abcam, Cambridge, UK). Secondary antibody HRP goat anti-mouse was obtained from Santa Cruz Biotechnology, Inc. and the goat anti-rabbit was purchased to Cell signalling. All others reagents were of analytical grade.

### *2.2. Plant material and preparation of SFT*

*Salvia fruticosa* plants, grown in an experimental farm located in Merelim, Braga, Portugal, were collected in June, 2004. The aerial parts of plants were air-dried and kept at -20°C with the accession number SF062004, under the responsibility of the Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Minho. Voucher specimen is also kept in an active bank in Braga, Portugal, also under the accountability of CITAB.

SFT was routinely prepared as formerly described (Lima et al., 2005) by pouring 150 ml of boiling water onto 2 g of the dried plant material and allowing it to steep for 5 min. The preparation produced a 2.8 ± 0.1 mg of extract dry weight per ml of infusion (0.28% w/v) and a yield of 19.1% w/v in terms of initial crude plant material of SF. Subsamples of freeze-dried extract (0.01 g) were redissolved in 1 ml of ultrapure Milli Q water and aliquots of 20 ml were injected into the HPLC/DAD system and analyzed as previously described (Santos-Gomes et al., 2002). RA (577.29 µg/ml), 6-hydroxyluteolin-7-glucoside (104.78 µg/ml) and a heteroside of unidentified flavones (99.13 µg/ml) were the most representative phenolic compounds (Azevedo et al., 2011).

*2.3. Animals*

Male Wistar rats (6 weeks old) were acquired from Charles River Laboratories (Barcelona, Spain) and kept in the authorized animal facilities of the Life and Health Sciences Research Institute (ICVS) from University of Minho. The animals were maintained under controlled temperature (20 ± 2ºC) and humidity (55 ± 10%) with a 12 h light/12 h dark cycle, and given food and tap water *ad libitum*. Animals were kept and handled in accordance with the NIH guidelines for the experimental use and care of laboratory animals by authorized investigators by the Direcção Geral de Veterinária (DGV), Portugal, and the experiment approved by the university's ethics committee that follows NIH guidelines (NIH Publication No.80-23; revised 1978) for the experimental use and care of laboratory animals.

*2.4. Experimental outline*

The experiment was conducted with thirty male rats that were subjected to dietary carbohydrate manipulation. Diet composition is presented in **Table 1**, where the normal rat chow (UAR-A04 chow diet, Reus, Spain) with 60.3% carbohydrates was considered the high carbohydrate (Hc) diet and the soybean diet with 28.0% of carbohydrates was referred as low carbohydrate (Lc) diet (soybean meal 47.5, Cargill S.A.C.I., Buenos Aires, Argentina, kindly supplied by NANTA, Fábricas de Moagem do Marco S.A., Marco de Canaveses, Portugal).

Animals were divided into five groups of six animals each: group 1 - rats fed with water and food (normal rat chow – referred as Hc diet) *ad libitum* for 14 days; group 2 – rats fed with water and food (Hc) *ad libitum* for 7 days, which followed a soybean diet (Lc diet) replacing the normal rat chow *ad libitum* for 7 additional days; group 3 – rats treated as the group 2, and afterwards fed for additional 4 days with water and Hc diet *ad libitum* (HcW group); group 4 – rats were treated as in group 2, followed by 4 additional days feeding with daily fresh SFT (replacing the water drinking) and Hc diet *ad libitum* (HcSFT group) and finally group 5 – rats treated as the group 4, with daily fresh RA solution replacing the SFT drinking (HcRA group).

**Table 1.** Composition of the different diets used in the study (approximately values in %).

| | Hc diet | Lc diet |
|---|---|---|
| **Carbohydrate** | 60.3 | 28.0 |
| **Protein** | 15.4 | 47.5 |
| **Fibre** | 4.1 | 4.2 |
| **Fat** | 2.9 | 2.0 |
| **Ash** | 5.3 | 6.3 |
| **Water** | 12 | 12 |

The RA solution was prepared by diluting RA in tap water to the same concentration found in the SFT (577 µg/ml). Food and beverage consumption, or animal body weight were not modified by the replacement of water by SFT or RA. The experimental outline is represented in **Figure 1**. At the end of the treatment, animals were sacrificed by decapitation and liver and intestinal mucosa samples were collected, frozen in liquid nitrogen and kept at -80ºC for further analyses. Blood samples were also collected to measure plasma insulin and lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides).

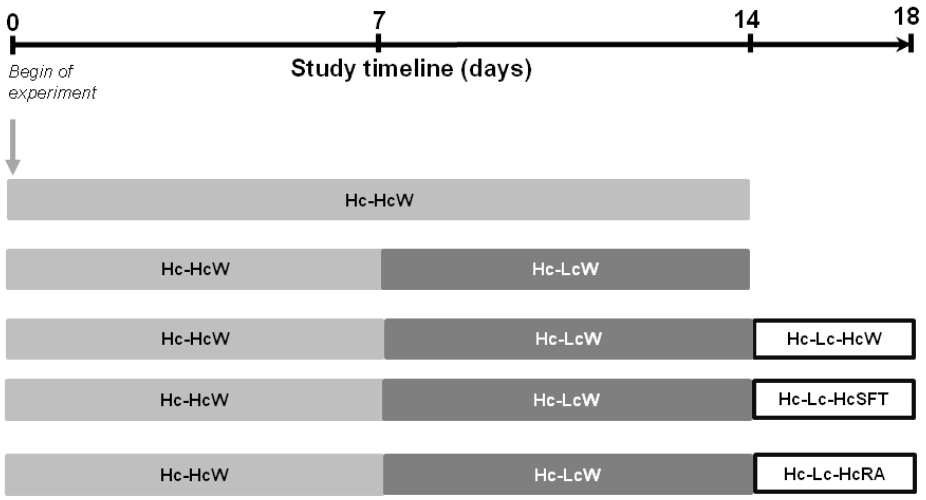


**Figure 1.** Experimental outline of the study. Animals were fed a high-carbohydrate (Hc) and/or a low-carbohydrate (Lc) diets and given either water (W), *Salvia fruticosa* extract (SFT) or rosmarinic acid (RA) to drink according to the figure.

*2.5. Characterization of lipid profile and hepatic triglyceride content*

Total cholesterol, LDL and HDL cholesterol and triglycerides levels were measured in rat plasma using spectrophotometric commercial kits (Spinreact), according to the manufacturer's specifications. Hepatic triglycerides were also measured by the same spectrophotometric procedure.

*2.6. Plasma insulin measurement*

Rat plasma insulin levels were measured using an ELISA-based commercial kit (Shibayagi Co.), following the manufacturer's specifications.

*2.7. RNA extraction and quantitative real-time PCR (qPCR)*

Total RNA was isolated from liver samples using Tri Reagent (Sigma-Aldrich), according to the manufacturer's recommended procedures. Concentrations and purity were verified by measuring optical density at 260 and 280 nm and 1 µg RNA was reversed transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. cDNA integrity was checked by agarose gel electrophoresis. Quantitative gene expression analysis was performed using SYBR Green technology (SsoFast EvaGreen supermix) and the CFX96 Real-Time system (both from Bio-Rad). All samples were amplified as duplicates using the following conditions: an initial denaturation step (3 min at 95°C) followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. Specific primers used were designed using Nucleotide BLAST tool of the National Center for Biotechnology Information (NCBI) and were manufactured by STAB VIDA (Portugal). Their sequences are described in **Table 2**. Each assay included a relative standard curve constructed from serial dilutions of cDNA from control samples. Target genes' transcript levels were all normalized to β-Actin mRNA levels and relative expression values of the control (Hc group) were set to 1.

*2.8. Liver homogenates*

A small amount of liver was homogenized in cold lysis buffer (0.5% NP-40 in 50 mM $Na_2HPO_4$, pH 7.4, 150 mM $NaCl_2$, 2 mM EDTA,) containing protease (1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin) and phosphatase (20 mM NaF, 20

mM $Na_3VO_4$) inhibitors, added just before use. The homogenate was then centrifuged at 10,000 × *g* at 4°C for 10 min and the supernatant collected. The amount of protein was measured by the Bio-Rad DC protein assay, following manufacturer instructions, using BSA as a standard.

**Table 2.** Primers used in this study for qPCR.

| Gene | Sequences | Product size (bp) | Efficiency |
|---|---|---|---|
| **SREBP-1** | *Sense:* AGCGCTACCGTTCCTCTAT<br>*Antisense:* GCGCAAGACAGCAGATTTAT | 95 | 2.10 |
| **SREBP-2** | *Sense:* ATTCCCTTGTTTTGACCACGC<br>*Antisense:* TGTCCGCCTCTCTCCTTCTTTG | 248 | 2.10 |
| **PPARα** | *Sense:* GATTCGGAAACTGCAGACCTC<br>*Antisense:* TAGGAACTCTCGGGTGATGA | 444 | 2.01 |
| **CPT1** | *Sense:* CAGGATTTTGCTGTCAACCTC<br>*Antisense:* GAGCATCTCCATGGCGTAG | 162 | 2.10 |
| **LDLR** | *Sense:* GCATCAGCTTGGACAAGGTGT<br>*Antisense:* GGGAACAGCCACCATTGTTG | 114 | 2.05 |
| **HMGCR** | *Sense:* AGTGATTGTGTCAGTATTATTGTGGAAG<br>*Antisense:* GGTACTGGCTGAAAAGTCACAA | 91 | 2.00 |
| **ChREBP** | *Sense:* CTGGTGTCTCCCAAGTGGAA<br>*Antisense:* CACCGCTGAAGAGGGAGTCAACCA | 700 | 2.42 |
| **β-Actin** | *Sense:* AGAGGGAAATCGTGCGTGAC<br>*Antisense:* CAATAGTGATGACCTGGCCGT | 138 | 2.04 |

*2.9. Isolation of brush-border membranes (BBMs)*

BBMs were isolated from frozen jejunal mucosal scrapings using a combination of cation precipitation and differential centrifugation as previously reported (Azevedo et al., 2011). BBMs were then frozen in liquid nitrogen and stored at -80ºC until use. Protein content was measured with the Bradford Reagent (Sigma-Aldrich), with BSA as a protein standard.

*2.10. Western blot analysis*

Twenty five to forty micrograms of protein of each sample were resolved in SDS-polyacrylamide gel and then transferred onto Hybond-P polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK). Membranes were blocked in 5% (w/v) non-fat dry milk in TPBS (0.05% (v/v) Tween 20 in PBS, pH 7.4), washed in TPBS and then incubated overnight with primary antibody. After washing, membranes were incubated with secondary antibody and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under an imaging densitometer, the ChemiDoc XRS (Bio-Rad). Band area intensity was quantified using the Quantity One software from Bio-Rad. β–Actin was used as loading control.

*2.11. HMGCR activity in vitro assay*

The effects of SFT and RA in the *in vitro* activity of the HMGCR enzyme were determined using a commercial kit from Sigma-Aldrich, following the manufacturer's specifications. RA was dissolved in DMSO (final concentration in the assays of 0.5% (v/v)), whereas SFT powder was prepared in water. To exclude possible effects, DMSO and water were used in the assay as controls. A statin (pravastatin, 0.5 μM) was used as an inhibitor control of the enzyme activity.

*2.12. Statistical Analysis*

Data are presented as means ± SEM. For statistical analysis, GraphPad Prism 4.0 software (San Diego, CA, USA) was used. Student's t-test was used to compare differences within the different carbohydrate groups (Hc-Hc, Hc-Lc and Hc-Lc-HcW groups) and to compare differences between the *in vitro* activity of HMGCR and the

compounds tested. One-way ANOVA followed by the Newman–Keuls multiple comparison test was employed to compare different drinking treatments (Hc-Lc-HcW, Hc-Lc-HcSFT and Hc-Lc-HcRA groups). Statistical significance was assumed for *P* values ≤ 0.05.

## 3. Results

### *3.1. SFT drinking but not RA increases plasma HDL cholesterol levels in rats fed a Hc diet*

As shown in **Figure 2** feeding the Lc diet for 7 days produced a significant reduction in plasma total cholesterol (16%), LDL (19%) and HDL cholesterol (16%) levels, whereas the reintroduction of the Hc diet returned all parameters, except the LDL cholesterol, to the control levels. SFT and RA treatment did not modify total cholesterol (**Figure 2A**) neither LDL cholesterol levels (**Figure 2B**). However, SFT significantly induced a significant enhancement of plasma HDL cholesterol levels (by 21%), compared to the control (**Figure 2C**). A slight decrease in LDL/HDL ratio was also observed in SFT group **(Figure 2D**).

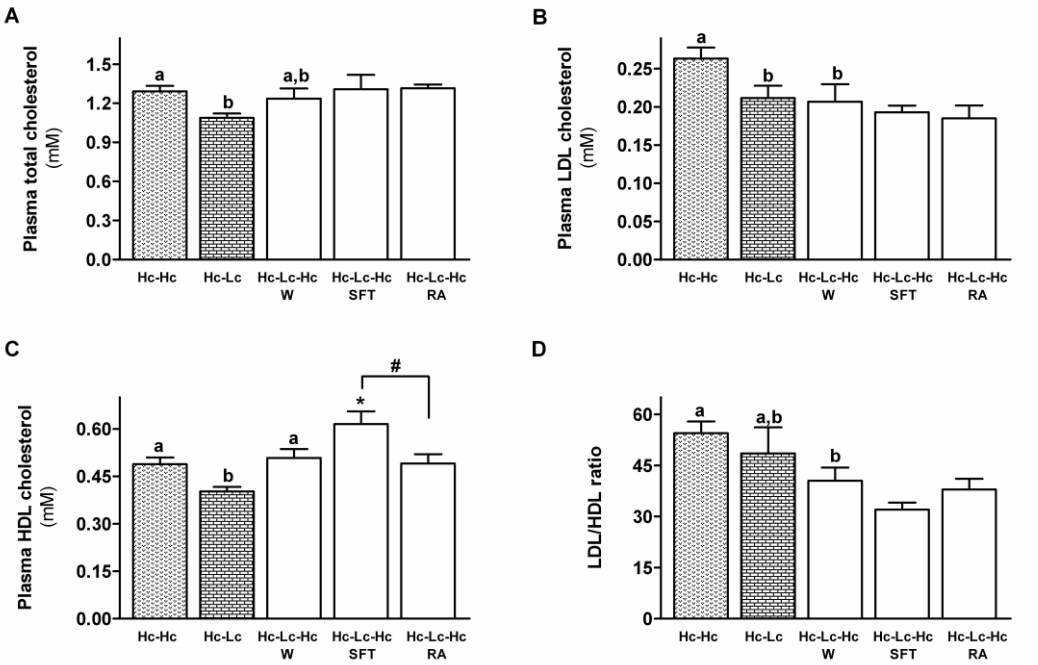


**Figure 2.** Effects of dietary carbohydrates, SFT and RA on rat plasma total cholesterol [**A**], LDL cholesterol [**B**], HDL cholesterol [**C**] and LDL/HDL ratio [**D**]. Values are means ± SEM of at least six animals. Effect of carbohydrate diet: groups with the same letter notation are not significantly different from each other ($P > 0.05$). Effect of beverage: $^{*}P \leq 0.05$ when compared to HcW group and $^{\#}P \leq 0.05$ when compared HcSFT and HcRA group.

Hepatic and plasma triglyceride levels were also measured in the rat liver homogenates. As depicted in **Figure 3A**, neither the carbohydrate composition of the diet nor the drinking of SFT or RA modified the hepatic triglyceride content of the studied animals. Conversely, plasma triglyceride levels increased significantly in animals submitted to the Lc diet (**Figure 3B**), while the reintroduction of the Hc diet reduced plasma triglyceride to levels lower than the control. Four days of the reintroduction of the Hc diet with SFT or RA in replacement of water, did not alter significantly plasma triglyceride levels (**Figure 3B**).

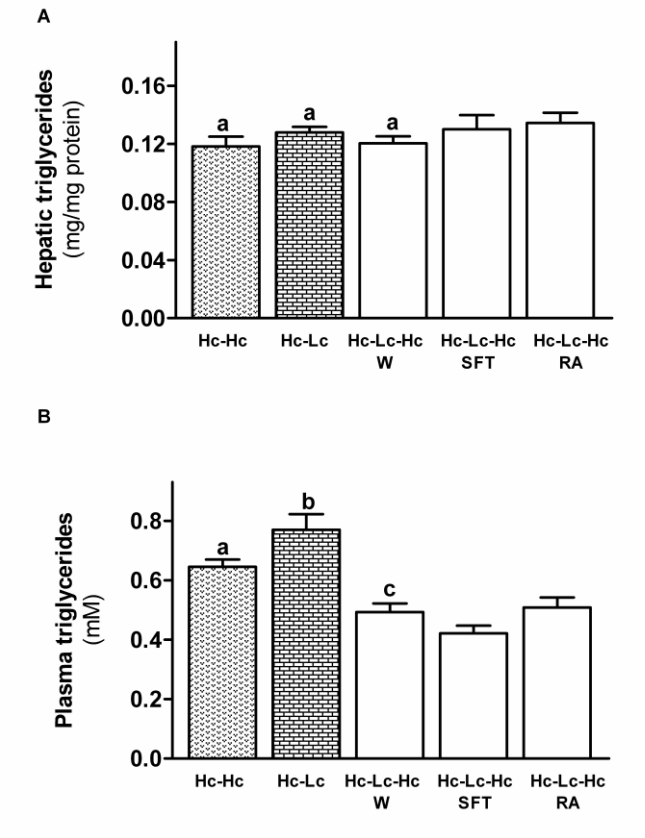


**Figure 3.** Hepatic [**A**] and plasma triglyceride [**B**] content of animals submitted to a dietary carbohydrate manipulation and to the drinking of SFT or RA. Values are means ± SEM of at least six animals. Effect of carbohydrate diet: groups with the same letter notation are not significantly different from each other ($P > 0.05$).

*3.2. SFT and RA drinking significantly affect plasma insulin levels in rats fed a Hc diet*

The animals fed the Lc diet showed a 56% reduction in plasma insulin levels when compared with the Hc group (**Figure 4**). The reintroduction of the Hc diet returned plasma insulin to control levels. In SFT and particularly RA treated animals, plasma insulin levels remained significantly low compared to the control HcW group (30% and 73% decline, respectively), as showed in **Figure 4**. This result supports our previous observations in which RA showed to prevent the return of plasma glucose to

control levels ($P \leq 0.01$), whereas SFT only showed to stabilize it (Azevedo et al., 2011).

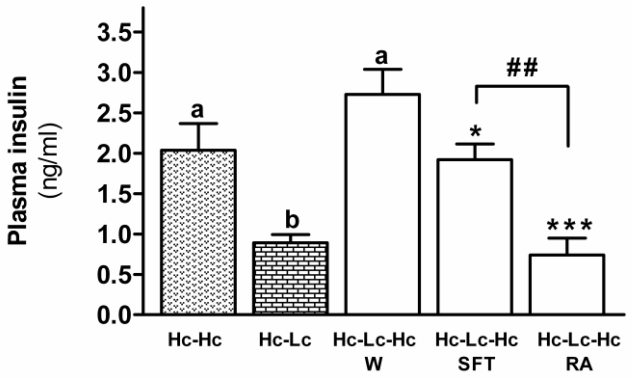


**Figure 4.** Effects of dietary carbohydrates, SFT and RA on rat plasma insulin. Values are means ± SEM of at least six animals. Effect of carbohydrate diet: groups with the same letter notation are not significantly different from each other ($P > 0.05$). Effect of beverage: $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$ when compared to HcW group. $^{\#\#}P \leq 0.01$ when compared HcSFT and HcRA group.

*3.3 RA significantly increases PPARα and CTP1 mRNA levels in rat hepatocytes*

In order to characterize the effects of both SFT and RA drinking in the animal's response to dietary carbohydrates, mRNA expression of several genes involved in lipid metabolism was analysed by quantitative real-time PCR. The Lc diet significantly affected only the SREBP-1 mRNA levels, where a 60% decline on gene expression was observed (**Figure 5A**). RA treatment not only inhibited the recovery but it almost abolished SREBP-1 mRNA expression (96% decline). RA also increased the expression of PPARα and CPT1 (in 3.1 and 5.1 fold, respectively), strongly indicating a role of RA on cellular lipolytic processes (**Figure 5B** and **C**). Moreover, and despite only slightly decreasing SREBP-2 gene expression (**Figure 5D**), 4 days of RA drinking showed to significantly decrease the mRNA levels of both HMGCR and LDLR genes (65% and 45% reduction, respectively) (**Figure 5E and F**). Four days of SFT drinking showed also to significantly reduce the HMGCR mRNA levels, by 39% (**Figure 5E**), but had no effects on PPARα, CPT1 and LDLR expression (**Figure 5B**, **C** and **F**). The ChREBP mRNA levels were however, significantly reduced by 4 days of SFT treatment (78% decline) (**Figure 5G**).

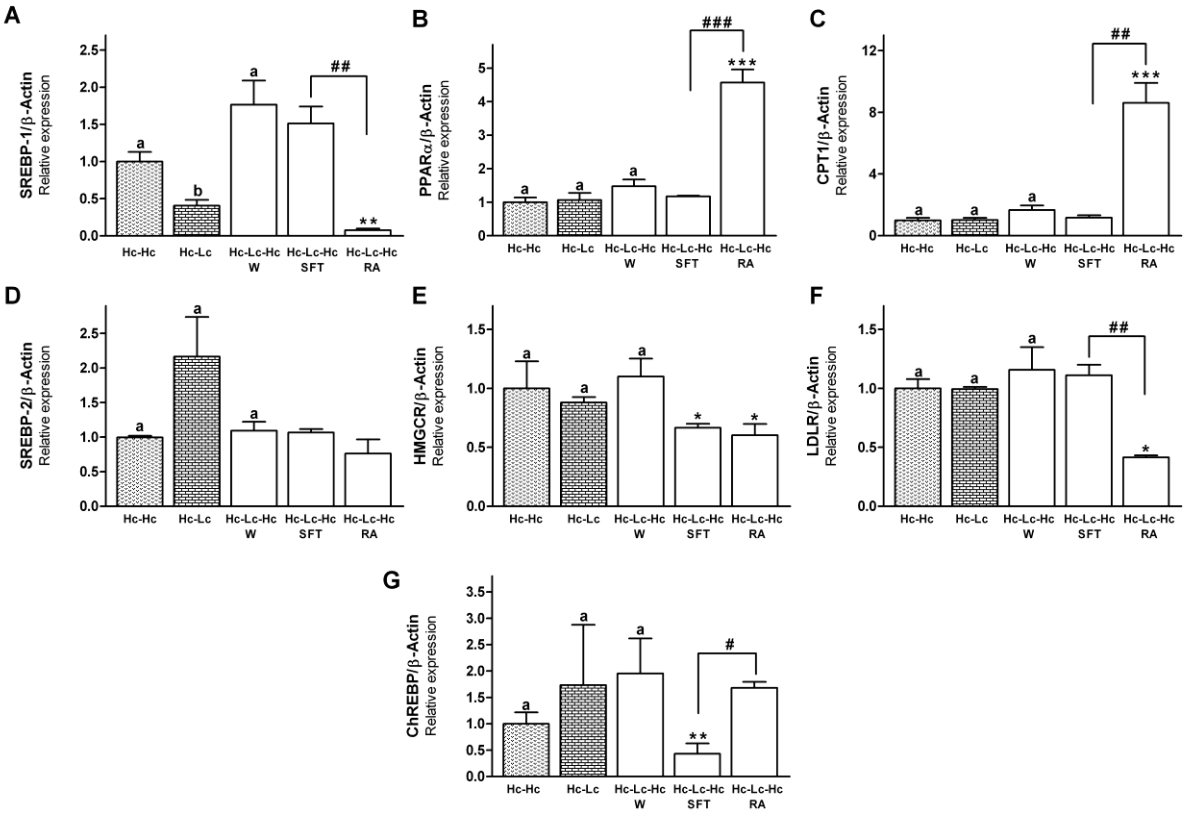


**Figure 5.** Effects of carbohydrate diet, SFT or RA treatment on SREBP-1 [**A**], PPARα [**B**], CPT1 [**C**], SREBP-2 [**D**], LDLR [**E**], HMGCR [**F**] and ChREBP [**G**] mRNA expression levels. Values are means ± SEM of the studied animals. Effect of carbohydrate diet: groups with the same letter notation are not significantly different from each other ($P > 0.05$). Effect of beverage: *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$ when compared to HcW group. $^{\#}$ $^{\#\#}P \leq 0.01$ and $^{\#}$ $^{\#}$ $^{\#\#}P \leq 0.001$ when compared HcSFT and HcRA group.

### *3.4. SFT and RA affect differently the expression pattern of proteins involved in hepatic lipid and glucose metabolism*

To determine whether SFT or RA affect the expression of proteins involved in fatty acid metabolism, the hepatic levels of total and phosphorylated ACC, as well as FAS proteins were studied by western blot. Lc diet induced a significant decrease in phospho-ACC/total ACC ratio and in FAS (75%) protein expression levels (**Figure 6A**, **B** and **D**). Four days of the reintroduction of the Hc diet (HcW group) did not bring phospho-ACC/total ACC protein ratio to the control (Hc group) levels and also modifications in the SFT and RA groups were not observed (**Figure 6A** and **D**). However, the expression of FAS protein increased with the reintroduction of Hc diet to levels exceeding the control (**Figure 6B** and **D**). FAS protein expression was

significantly reduced with both SFT (26%) and particularly with RA treatments (42%) (**Figure 6B** and **D**). Since AMPK (AMP-activated protein kinase) is a modulator of ACC and FAS activity, total and phosphorylated AMPK expression levels were also assayed however, changes were not observed (data not shown).

As expected, the Lc diet significantly increased the protein expression levels of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK), and 4 days of the reintroduction of the Hc diet returned almost completely PEPCK to control levels (**Fig. 6C** and **D**). The effect was more pronounced in the SFT group (**Figure 6C** and **D**).

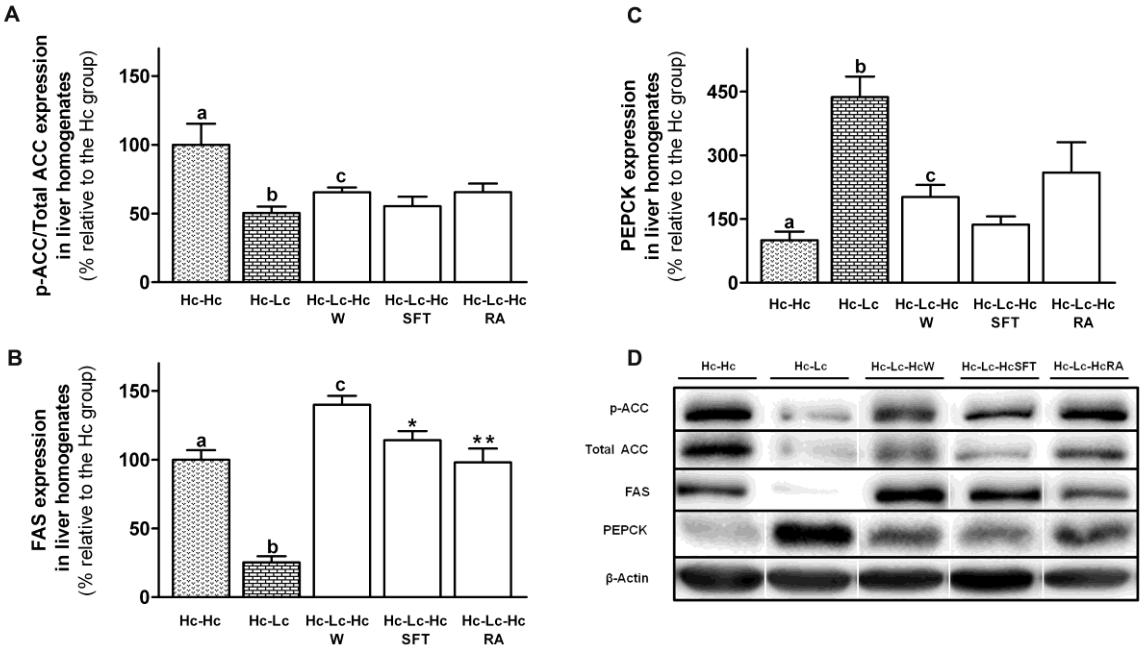


**Figure 6.** Western blot analysis of phosphorylated and total ACC [**A**], FAS [**B**] and PEPCK [**C**] proteins in rat liver homogenates. Representative blots and corresponding loading control (β-Actin) of the experiments [**D**]. Values are means ± SEM of at least six animals. Effect of carbohydrate diet: groups with the same letter notation are not significantly different from each other ($P > 0.05$). Effect of beverage: *$P \leq 0.05$ and **$P \leq 0.01$, when compared to HcW group.

### *3.5. RA affects the expression levels of the NPC1L1 transporter at BBM*

In order to determine the effects on intestinal cholesterol absorption, the BBM expression of NPC1L1 protein was assayed. RA significantly reduced the expression of NPC1L1 protein after the reintroduction of the Hc diet (**Figure 7A and C**). Both SFT and RA drinking as well as the dietary carbohydrate manipulation did not seem to affect the intestinal epithelial proliferation, since no effects on the expression of proliferating

cell nuclear antigen (PCNA) in intestinal homogenates were observed (**Figure 7B and D**).

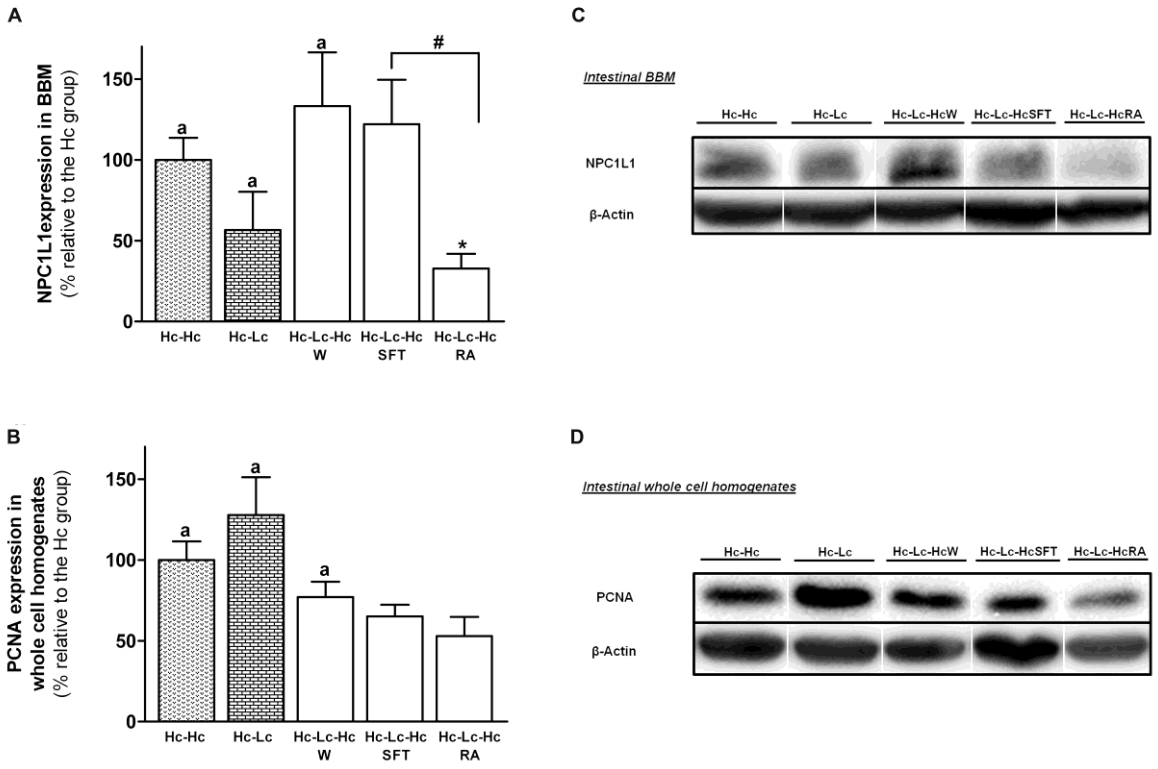


**Figure 7.** Western blot analysis of NPC1L1 transporter [**A**] at the enterocytic BBM and PCNA [**B**] protein in intestinal whole cell homogenates. Representative blots and corresponding loading control (β-Actin) of the experiments [**C** and **D**]. Values are means ± SEM of at least six animals. Effect of carbohydrate diet: groups with the same letter notation are not significantly different from each other ($P > 0.05$). Effect of beverage: $^{*}P \leq 0.05$ when compared to HcW group and $^{\#}P \leq 0.05$ when compared HcSFT and HcRA group.

### *3.6. RA reduces significantly the in vitro activity of HMGCR*

In order to determine the ability of SFT and RA to inhibit cholesterol endogenous synthesis, their effects on the *in vitro* activity of HMGCR were determined. A significant decrease of the enzyme' activity was observed for 100 µM and particularly for 10 µM of RA (32% and 40% decline, respectively) (**Figure 8**). Although not significant, SFT showed a similar inhibition pattern to RA for the 10 and for 100 µM extract concentrations (with 28% and 24% reduction, respectively) (**Figure 8**). However, the pharmacological drug, pravastatin (0.5 µM), showed to be the most effective inhibitor of the *in vitro* activity of HMGCR (80% inhibition) (**Figure 8**).

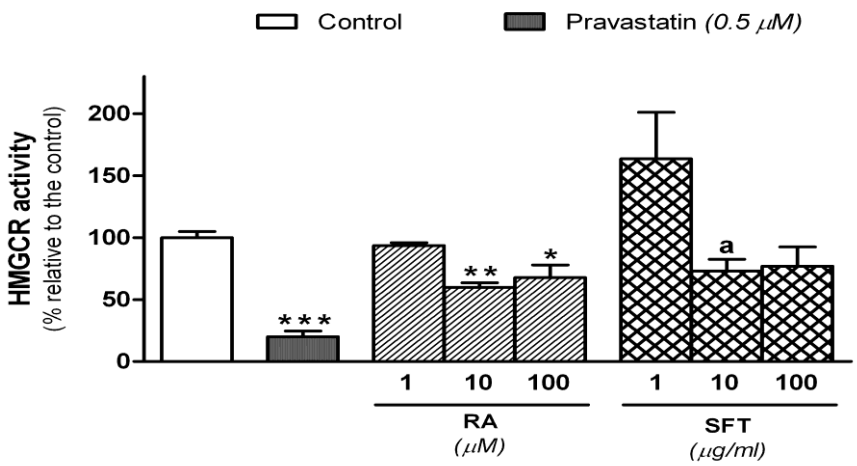


**Figure 8.** Effects of RA and SFT on HMGCR *in vitro* activity. Concentrations are expressed in μM for RA and μg/ml for SFT. Pravatatin (0.5 μM) was used as an inhibitor of HMGCR activity. Values are means ± SEM, n=3. $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.001$ when compared to the control. $^{a}P = 0.06$ when compared with the control.

## Discussion

The present study demonstrated that SFT drinking promote an enhancement on plasma HDL cholesterol levels in animals fed a Hc diet. The mechanism behind this beneficial effect still poorly understood and demand further investigations however, our data arises promising since there are limited available agents for increasing the levels of these lipoproteins (Greenfeder, 2009). In addition, the lack of effects of both SFT and RA drinking on plasma triglyceride, total and LDL cholesterol and hepatic triglyceride levels may suggest that treatment for longer periods of time may be required to verify changes at these levels.

We have recently reported that two weeks of SFT drinking stabilized fasting blood glucose levels without affecting plasma insulin or liver glycogen content in streptozotocin-diabetic rats (Azevedo et al., 2011). This study also revealed that, while RA drinking reduced plasma glucose levels, SFT and particularly RA drinking significantly prevented the carbohydrate-induced adaptive increase of the BBM SGLT1 protein (Azevedo et al., 2011). The study here presented showed that SFT and more particularly RA, prevented the return of plasma insulin to control levels, during 4 days of the reintroduction of the Hc diet. Fructose-fed hypertensive rats were recently used to demonstrate that RA may be an effective substance in preventing insulin resistance and consequently CVDs onset. The authors found that RA supplementation reduced

oxidative and myocardial injury, enhanced insulin sensitivity and diminished lipid levels in those animals (Karthik et al., 2011). RA also showed to improve insulin resistance by ameliorating the redox status of fructose-fed mice (Vanithadevi and Anuradha, 2008). Thus, the observed effects of both phytochemicals on insulin levels in response to dietary carbohydrates may prevent insulin resistance and thus delay or even repress several pathologies, including the MetS.

A significant reduction of the hepatic FAS protein levels, the required enzyme for *de novo* lipogenesis was also mediated by both SFT and RA. Several reports showed that FAS expression is highly regulated at the transcriptional level through the combined action of ChREBP and SREBP-1 in response to glucose and insulin, respectively (Shimomura et al., 1999a, Dentin et al., 2005). In addition, 4 days of RA drinking showed to effectively decrease SREBP-1 gene expression, while 4 days of SFT treatment showed to diminish ChREBP mRNA levels. Considering these evidences, we suggest that fatty acid synthesis is prevented by two distinct mechanisms: through suppressing SREBP-1 expression (via RA) and by preventing ChREBP gene expression raises (via SFT) in response to dietary carbohydrates.

Interestingly, RA significantly stimulated the mRNA levels of the peroxisome proliferator-activated receptor alpha (PPARα), a transcription factor highly expressed in tissues with high metabolic rates such as the liver, heart, skeletal muscle and the kidney (Mandard et al., 2004). Since PPARα plays a central role in the regulation of hepatic lipid metabolism by modulating fatty acid oxidation, lipoprotein metabolism, PPARα agonists are considered attractive potential therapeutic agents for preventing the development of lipid-related diseases (Kersten et al., 1999, Burri et al., 2010). In addition, RA also stimulated the hepatic mRNA levels of CPT1 suggesting that this phytochemical may act as a PPARα agonist and activates the transcription of lypolitic genes involved in fatty acid oxidation, and thus preventing fatty acid synthesis.

The BBM cholesterol transporter NPC1L1 was significantly affected by RA drinking with the reintroduction of the Hc diet, indicating that this phenolic acid may inhibit the dietary cholesterol absorption, a similar outcome of the pharmacological drug ezetimibe. It has been reported that ezetimibe binds to intestinal BBM vesicles that contains NPC1L1 and does not bind to BBM vesicles from NPC1L1-deficient mice (Garcia-Calvo et al., 2005). Through suppressing the intestinal cholesterol absorption via NPC1L1 pathway RA, like ezetimibe, may not only reduce the delivery of fatty acids from the gut to extrahepatic tissues and cholesterol to the liver by the chylomicron

pathway and decrease the hepatic cholesterol secretion into bile (Wang et al., 2008), but also prevent intestinal fatty acid absorption (Labonte et al., 2008, de Bari et al., 2011). As showed in NPC1L1 knockout mice and in ezetimibe-treated animals, reduced cholesterol absorption leads to increased fecal cholesterol excretion and to endogenous cholesterol biosynthesis (Altmann et al., 2004, Davis et al., 2004). Patients under ezetimibe treatment prevented intestinal cholesterol absorption, an effect accompanied by a reduction on plasma LDL levels and a compensatory increase on hepatic cholesterol biosynthesis (Sudhop and von Bergmann, 2002). Therefore, the combined administration of ezetimibe and HMGCR inhibitors, like statins, offers a more efficient reduction in plasma total and LDL cholesterol and a powerful approach to prevent atherosclerosis onset (Gagne et al., 2002, Kerzner et al., 2003, Melani et al., 2003, Grigore et al., 2008). Both SFT and RA significantly decreased the hepatic HMGCR mRNA levels, whereas RA inhibited the hepatic *in vitro* activity of this enzyme. Additionally, RA also repressed the mRNA levels of the hepatic LDLR however, it only promoted a slight decrease on the SREBP-2 gene expression. The precise mechanism responsible for these evidences is under investigation however, our data suggests that RA is not the active principle responsible for the reported SFT-mediated effects since both natural products showed to differently affect *de novo* lipogenesis in response to dietary carbohydrates.

The findings here reported not only proposed a link between glucose and lipid metabolism, but also demonstrated the beneficial effects of SFT and RA in preventing both intestinal glucose and cholesterol uptake and promoting fat oxidation in the liver, in response to dietary carbohydrates. Comparing the effects of these natural products with the lipid-lowering available drugs must be considered in further researches to evaluate their efficacy and safety, and may open the door to the finding of new pharmacological interventions that delay the onset of the MetS and its related pathologies and improve life quality.

**Acknowledgements**

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# CHAPTER 4

## *Exploring the lipid-lowering effects of luteolin-7-glucoside*

## 4.1. Chapter overview

Our recent findings demonstrated that L7G, a phytochemical particularly abundant in *Salvia* species, ameliorate the lipid profile of healthy animals by lowering plasma total and LDL cholesterol levels[1]. In this chapter we reported the potential mechanisms involved on the improvement of lipid profile and metabolism mediated by L7G.

L7G treatment induced a significant increase on PPARα mRNA levels and its target gene CPT1, and showed a slight tendency to decrease SREBP-1 gene expression in the liver. Findings on protein levels of FAS, ACC and GRP78/BIP suggest the potential role of L7G in blocking SREBP-1 maturation and/or activity and ER stress activation in the liver, without activating proapoptotic signals. A significant reduction of HMGCR gene expression was mediated by L7G-supplemented diet. Moreover, the *in vitro* activity of the HMGCR enzyme was also repressed by L7G in a dose-dependent way however, pravastatin showed to be the most effective inhibitor of this enzyme activity.

The potential lipid-lowering properties of L7G should be tested in further experiments however, the data here reported add promising evidences to this assumption.

[1] Azevedo MF, Camsari C, Sa CM, Lima CF, Fernandes-Ferreira M and Pereira-Wilson C (2010) Ursolic acid and luteolin-7-glucoside improve lipid profiles and increase liver glycogen content through glycogen synthase kinase-3. *Phytother Res,* **24 Suppl 2**: S220–S224.

## 4.2. Manuscript 2

This chapter comprises the following manuscript:

**Carla M Sá**, Marisa Azevedo and Cristina Pereira-Wilson (**2012**). The mechanisms behind the *in vivo* lipid-lowering effects of luteolin-7-glucoside (*in preparation*).

# The mechanisms behind the *in vivo* lipid-lowering effects of luteolin-7-glucoside

Carla M. Sá[1], Marisa Azevedo[1] and Cristina Pereira-Wilson[1]

[1] *CBMA - Centre of Molecular and Environmental Biology/ Department of Biology, University of Minho, 4710-057 Braga, Portugal.*

## Abstract

Lipids are crucial molecules for many biological processes in multicellular organisms. However, disruptions in whole-body lipid metabolism can lead to the onset of several pathologies such as cardiovascular diseases (CVDs) and type 2 diabetes mellitus (T2DM). The present work aimed to elucidate the molecular mechanisms behind the lipid-lowering effects of the flavonoide luteolin-7-glucoside (L7G) which showed to, further than a tendency to promote glycogen synthesis in the liver, reduce plasma glucose levels and improve plasma lipid profile in healthy rats. A potential role of L7G on regulating lipolytic processes such as fatty acid oxidation was suggested by the observed increase of PPARα and carnitine palmitoly transferase 1 (CPT1) gene expression. Dietary supplementation with L7G showed not only a tendency to decrease the hepatic gene expression of sterol regulatory element-binding protein-1 (SREBP-1), without affecting SREBP-2 mRNA levels, but also failed to induce the hepatic protein levels of FAS or ACC. A simultaneous upregulation of the 78 kDa glucose-regulated protein/immunoglobulin-binding protein (GRP78/BIP) chaperone was observed in animals submitted to L7G-supplemented diet, with no induction of proapoptotic signals through JNK or caspase-3 activation. Moreover, the HMG CoA reductase (HMGCR) gene expression was repressed by L7G, which was also able to inhibit the *in vitro* activity of this enzyme in a dose-dependent manner, albeit to a smaller extent than the conventional pravastatin. Convinced that more studies are required to the complete elucidation of the role of L7G on lipid metabolism, the work described herein added new evidences to our believe that including this phytochemical in diet may prevent T2DM and CVDs progression and contribute to general health improvement.

**Key words:** Luteolin-7-glucoside – SREBP – PPARα – HMGCR

## 1. Introduction

In multicellular organisms, lipids function as energy stores, are the major structural components of cellular membranes and precursors of vitamins, bile acids and steroid hormones (Desvergne et al., 2006; Eberle et al., 2004). Daily lipid requirements are obtained in diet or endogenously produced in a coordinated process that ensures lipid homeostasis. The endogenous synthesis of fatty acids and cholesterol in the liver is regulated by the sterol regulatory element-binding proteins (SREBPs) (Horton et al., 2002). SREBPs are considered key regulators of lipogenesis since they are responsible for controlling fatty acid biosynthesis (SREBP-1) or regulating cellular cholesterol synthesis and uptake (SREBP-2) (Osborne, 2000; Peschel et al., 2007). When the intracellular sterol concentration is low, the inactive endoplasmatic reticulum (ER) membrane-bound SREBPs precursors are escorted to Golgi apparatus for proteolytical cleavage (Eberle et al., 2004). Once activated, SREBPs are translocated to the nucleus where it binds to sterol regulatory elements (SREs) and activates the transcription of several target genes including the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and the LDL receptor (LDLR) (via SREBP-2) or the acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (via SREBP-1) (Amemiya-Kudo et al., 2002). SREBP-dependent lipogenesis is suppressed through AMP-activated protein kinase (AMPK)-dependent phosphorylation (Li et al., 2011). In addition, AMPK also increases the phosphorylation and consequent inactivation of ACC, leading to the inhibition of fatty acid synthesis and to the promotion of fatty acid oxidation through carnitine palmitolytransferase 1 (CPT1) activation (McGarry and Brown, 1997; Zhou et al., 2001). AMPK is also critical for the regulation of cholesterol synthesis since it phosphorylates and inactivates the rate-limiting enzyme of the process, the HMGCR (Clarke and Hardie, 1990; Corton et al., 1994; Henin et al., 1995; Steinberg and Kemp, 2009).

Despite all the crucial biological functions of lipids, imbalances in lipid homeostasis are strongly related to many pathological processes such as obesity, inflammation, cardiovascular diseases (CVDs), insulin resistance and type 2 diabetes mellitus (T2DM) (Pahan, 2006; Vergès, 2005). Diet is an important risk factor of these diseases but it may also contain constituents such as flavonoids, which have biological

properties, that can be used as potential therapeutic agents or to inspire the design of novel synthetic substances with improved pharmacological properties.

We have recently demonstrated that the consumption of luteolin-7-glucoside (L7G), an abundant compound in plants of the genus *Salvia* (family Laminaceae) but also present in vegetables like celery, green pepper and lettuce (Arabbi et al., 2004; Kim et al., 2006b; Lin et al., 2007), ameliorates the lipid profile of healthy rats (Azevedo et al., 2010). Dietary L7G is normally biotransformed at the intestinal level in its aglycone luteolin (3',4',5,7-tetrahydroxyflavone), a widely spread flavonoide present in several plants ranging from edible to medicinal herbs (Lopez-Lazaro, 2009), to which antioxidant (Lima et al., 2006) and anticarcinogenic (Chang et al., 2005; Ko et al., 2002) properties have been attributed. The *in vitro* lipid-lowering potential of luteolin has been recently demonstrated by the reduction of SREBP-1c and FAS gene expression and the increase of CPT1 expression in HepG2 probably owed to the activation of the AMPK pathway and its antioxidant properties (Liu et al., 2010).

Despite being more abundant in diet, little is known about the effects of the glycosilated form on the regulation of lipid metabolism. Therefore, the present work aimed to explore the mechanisms behind the previous observed lipid-lowering effect of L7G-supplemented diet in healthy rats.

## 2. Materials and Methods

### *2.1 Reagents and antibodies*

Anti-β-Actin antibody, Bradford reagent, Tri Reagent (Trizol eq.), HMGCR assay kit as well as all other reagents were from Sigma-Aldrich (St. Louis, MO, USA), unless afore mentioned. Luteolin-7-*O*-glucoside was acquired from Extrasynthese (Genay, France). Antibodies against phospho-ACC, total ACC, phospho-Akt, total Akt, FAS and phospho-AMPK, were purchased from Cell signalling (Danvers, MA, USA). The anti-AMPK total, anti-phospho-JNK, anti-JNK total and anti-caspase-3 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary antibodies HRP goat anti-mouse was acquired from Santa Cruz Biotechnology, Inc. and the goat anti-rabbit was purchased to Cell signalling. All others reagents were of analytical grade.

*2.2. Animals*

Male Wistar rats (6 weeks old) were acquired from Charles River Laboratories (Barcelona, Spain) and kept in the authorized animal facilities of the Life and Health Sciences Research Institute from University of Minho. The animals were maintained under controlled temperature (20 ± 2ºC) and humidity (55 ± 10%) with a 12 h light/12 h dark cycle, and given food and tap water *ad libitum*. Animals were kept and handled in accordance with the NIH guidelines for the experimental use and care of laboratory animals by authorized investigators by the Direcção Geral de Veterinária, Portugal, and the experiment approved by the university's ethics committee that follows NIH guidelines (NIH Publication No.80-23; revised 1978) for the experimental use and care of laboratory animals.

*2.3. Experimental design*

Fifteen male rats were used and divided into two groups: control and L7G-supplemented diet (**Figure 1**). The compound was mixed in a small piece of food and administered orally, once a day, for 7 consecutive days. Control group received vehicle only. The daily administrated dose (2 mg of L7G per kg of animal body weight) was based on estimations of physiological concentrations (Hertog et al., 1993). Water was given *ad libitum* to the animals during the experiment and the administration of L7G did not alter animal's body weight (compared to control group) nor modified animal's food and beverage consumption. Animals were sacrificed by decapitation and liver samples were collected, frozen in liquid nitrogen and kept at -80ºC for further analysis.

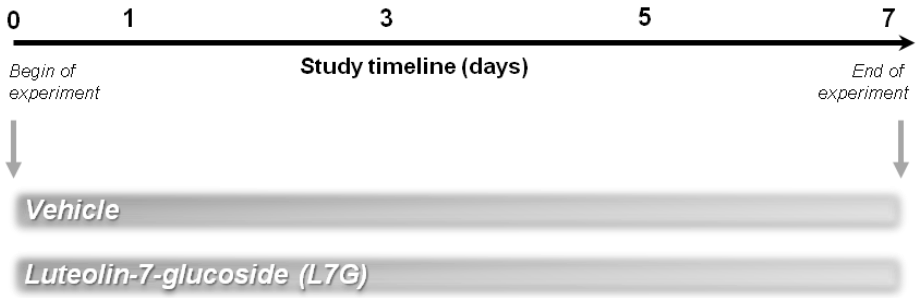


**Figure 1.** Schematic representation of the experimental design of the study.

*2.4. RNA extraction and quantitative real-time PCR (qPCR)*

Total RNA was extracted from liver samples using Tri Reagent (Sigma-Aldrich), following the manufacturer's recommendations. RNA concentrations and purity were confirmed by measuring optical density (at 260 and 280 nm) and RNA was converted into cDNA using iScript cDNA Synthesis kit from Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's recommended protocol. The integrity of cDNA was checked by agarose gel electrophoresis and the quantitative gene expression analysis was performed using SYBR Green technology (SsoFast EvaGreen supermix, Bio-Rad) and the CFX96 Real-Time system (Bio-Rad). The thermal cycler program was as follows: an initial denaturation step (3 min at 95°C) followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. The primers used, which sequences are described in **Table 1**, were designed using the Nucleotide BLAST tool of the National Center for Biotechnology Information (NCBI) and were manufactured by STAB VIDA (Portugal). Each assay included a relative standard curve constructed from serial dilutions of cDNA from control samples. Target genes' transcript levels were all normalized to β-Actin mRNA levels and expression values in the control were set to 1.

*2.5. Liver homogenates*

A small amount of liver was homogenized in cold lysis buffer (0.5% NP-40 in 50 mM $Na_2HPO_4$, pH 7.4, 150 mM $NaCl_2$, 2 mM EDTA,) containing protease (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) and phosphatase (20 mM NaF, 20 mM $Na_3VO_4$) inhibitors added just before use. The homogenate was then centrifuged at 10,000 × *g* at 4°C for 10 min and the supernatant collected. The amount of protein was measured using DC protein assay kit (Bio-Rad) following manufacturer instructions, using BSA as a standard.

*2.6. Western blot analysis*

Twenty five to forty micrograms of protein of each sample were resolved in SDS-polyacrylamide gel and then transferred onto Hybond-P polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK). Membranes were blocked in 5% (w/v) non-fat dry milk in TPBS (0.05% (v/v) Tween 20 in PBS, pH 7.4), washed in TPBS and then incubated overnight with primary antibody. After washing, membranes

were incubated with secondary antibody and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under an imaging densitometer, the ChemiDoc XRS (Bio-Rad). Band area intensity was quantified using the Quantity One software from Bio-Rad. β–Actin was used as loading control.

**Table 1.** Primers used in this study for qPCR analysis.

| Gene | Sequences | Product size (bp) | Efficiency |
|---|---|---|---|
| **SREBP-1** | *Sense:* AGCGCTACCGTTCCTCTAT<br>*Antisense:* GCGCAAGACAGCAGATTTAT | 95 | 2.10 |
| **SREBP-2** | *Sense:* ATTCCCTTGTTTTGACCACGC<br>*Antisense:* TGTCCGCCTCTCTCCTTCTTTG | 248 | 2.10 |
| **PPARα** | *Sense:* GATTCGGAAACTGCAGACCTC<br>*Antisense:* TAGGAACTCTCGGGTGATGA | 444 | 2.01 |
| **CPT1** | *Sense:* CAGGATTTTGCTGTCAACCTC<br>*Antisense:* GAGCATCTCCATGGCGTAG | 162 | 2.10 |
| **LDLR** | *Sense:* GCATCAGCTTGGACAAGGTGT<br>*Antisense:* GGGAACAGCCACCATTGTTG | 114 | 2.05 |
| **HMGCR** | *Sense:* AGTGATTGTGTCAGTATTATTGTGGAAG<br>*Antisense:* GGTACTGGCTGAAAAGTCACAA | 91 | 2.00 |
| **β-Actin** | *Sense:* AGAGGGAAATCGTGCGTGAC<br>*Antisense:* CAATAGTGATGACCTGGCCGT | 138 | 2.04 |

### *2.7. HMGCR activity in vitro assay*

In order to determine the potential for L7G affecting the endogenous cholesterol synthesis, we measured the *in vitro* activity of HMGCR enzyme, using a commercial kit from Sigma-Aldrich, following the manufacturer's specifications. L7G was dissolved in DMSO (final concentration in the assays of 0.5% (v/v)) and DMSO was used in the assay as control. A commercial statin (pravastatin, 0.5 μM) was used, as a negative control.

*2.8. Statistical Analysis*

Data are presented as means ± SEM. For statistical analysis, GraphPad Prism 4.0 software (San Diego, CA, USA) was used. Student's t-test was used to compare differences between control and L7G treatment. *P* values ≤ 0.05 were considered statistically significant.

## 3. Results

*3.1. L7G significantly increased liver PPARα and CPT1 gene expression, while decreasing HMGCR mRNA levels*

Quantitative real-time PCR was performed to evaluate the effects of L7G-supplemented diet on some genes involved in lipid metabolism. Although it showed a slight tendency to decrease SREBP-1 mRNA levels (**Figure 2A**), L7G significantly increased the hepatic mRNA levels of PPARα (**Figure 2B**) and its target gene CPT1 (**Figure 2C**) by 1.9 and 1.8 fold, respectively. Moreover, L7G reduced the hepatic HMGCR gene expression (**Figure 2F**), without significantly changing SREBP-2 and LDLR mRNA levels (**Figure 2D** and **E**, respectively).

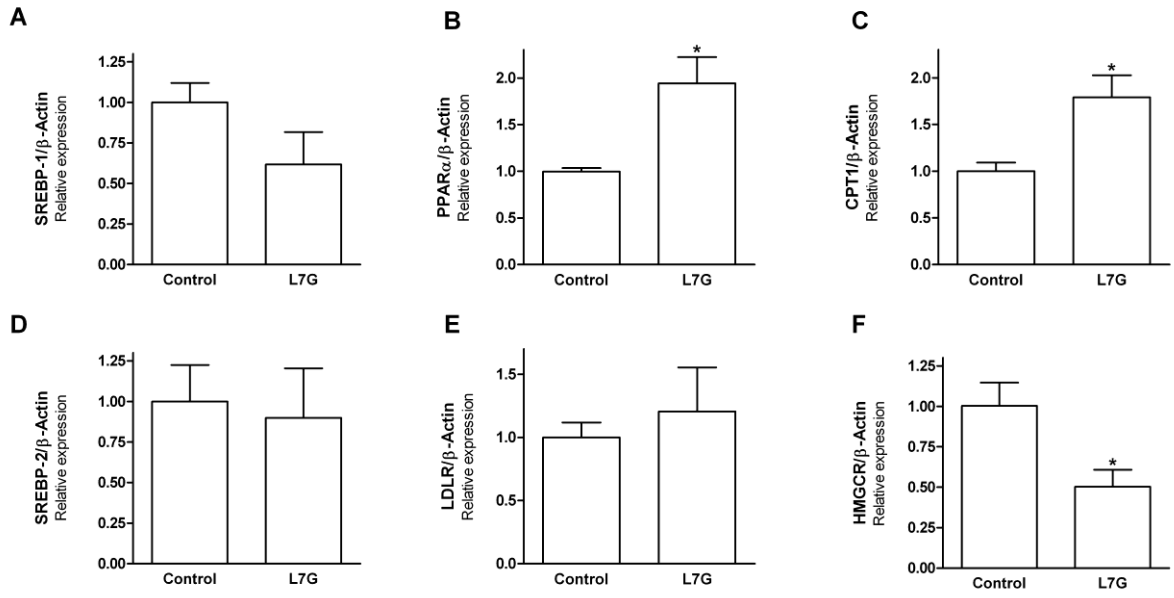


**Figure 2.** The effects of L7G-supplemented diet on SREBP-1 [**A**], PPARα [**B**], CPT1 [**C**], SREBP-2 [**D**], LDLR [**E**] and HMGCR [**F**] gene expression.Values are means ± SEM, n=5. **P* ≤ 0.05 when compared with the control group.

*3.2. L7G modified differently the hepatic expression of proteins involved in lipid metabolism*

As illustrated in **Figure 3A** and **D**, L7G significantly decreased the hepatic phospho-ACC/total ACC ratio (17% decline), without affecting the protein levels of FAS (**Figure 3B** and **D**). This decline results not from a decrease on AMPK activation mediated by L7G (data not shown), but from an increase on total ACC protein (**Figure 3D**), which may be explained by the potential role of this phytochemical to decrease the proteolytic cascade or to stimulate the synthesis of this protein.

The hepatic protein levels of 78 kDa glucose-regulated protein/immunoglobulin binding protein (GRP78/BIP), a Hsp70 chaperone located in the lumen of the ER, increased 1.53 fold with L7G treatment (**Figure 3C** and **D**).

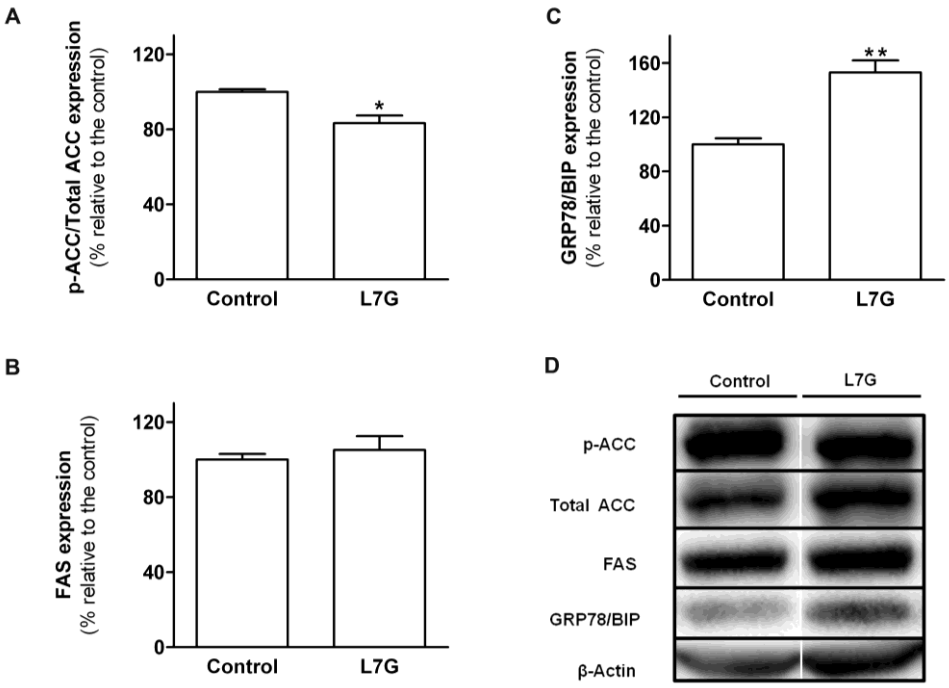


**Figure 3.** Western blot analysis of the hepatic phosphorylated and total ACC [**A**)], FAS [**B**] and GRP78/BIP chaperone [**C**] in animals treated with L7G-supplemented diet, with representative immunoblots and corresponding loading control (β-Actin) [**D**]. Values are means ± SEM, n=5. *$P \leq 0.05$ and **$P \leq 0.01$ when compared with the control group.

Diet-supplemented with L7G did not change Akt expression nor did lead to JNK or caspase-3 activation (data not shown), withdrawing a possible induction of insulin-resistance and/or proapototic signals in healthy rats.

*3.3. L7G significantly inhibited the in vitro activity of HMGCR enzyme*

The *in vitro* activity of the HMGCR enzyme was significantly inhibited by L7G treatment in a dose-dependent way, achieving the maximal inhibitory potential with the 100 μM concentration (***P* ≤ 0.01) (**Figure 4**). However, the most effective inhibitor of HMGCR *in vitro* activity was pravastatin (****P* ≤ 0.001) (**Figure 4**).

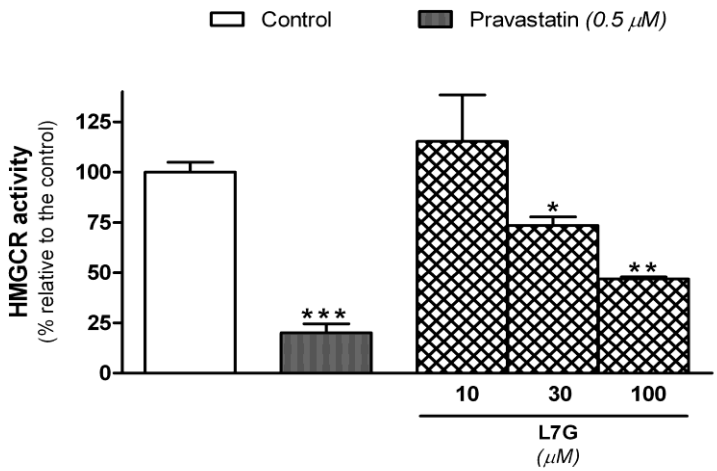


**Figure 4.** Effect of L7G on the HMGCR *in vitro* activity. Pravastatin was used as an inhibitor of the enzyme activity. Concentrations of L7G and the inhibitor are expressed in μM. Values are means ± SEM, n=3. **P* ≤ 0.05, ***P* ≤ 0.01 and ****P* ≤ 0.001 when compared to the control.

## Discussion

The present work aimed to clarify the precise mechanism behind the previously observed lipid-lowering and health-improvement properties of L7G, which showed to decrease plasma total and LDL cholesterol levels and plasma glucose levels, while demonstrating a tendency to promote hepatic glycogen deposition possibly through glycogen synthase kinase-3 (GSK3) inhibition (Azevedo et al., 2010).

Diet supplemented with L7G showed to significantly increase the mRNA expression of PPARα, a major regulator of the hepatic lipid metabolism, and its target gene CPT1. More than a promoter of fatty acid catabolism, ketogenesis, lipid transport and gluconeogenesis (Bernal-Mizrachi et al., 2003; Reddy and Hashimoto, 2001), PPARα also regulates the metabolism of lipoproteins through increasing HDL and decreasing LDL and VLDL levels (Fruchart et al., 1999). Thereby, this induction of PPARα expression may not only explain the previously observed decrease of plasma LDL cholesterol in these animals, but also suggest a potential role for L7G promoting fatty acid oxidation instead of fatty acid synthesis.

It has been recently demonstrated that fibrates and thiazolidinediones, agonists of PPARα and PPARγ respectively, reduced triglyceride synthesis in rat hepatoma through repressing SREBP-1 activation via up-regulation of Insig, a membrane protein of the ER that retains SCAP-SREBP complexes within this organelle (Konig et al., 2009). In addition, the up-regulation of GRP78/BIP, a major cellular target of the unfolded protein response (UPR) of the ER, also showed to reduce ER stress markers and repress SREBP-1c cleavage and the expression of SREBP-1c and SREBP-2 target genes in mice (Kammoun et al., 2009). The UPR is a biological adaptive response, activated through the accumulation of unfolded or misfolded proteins in the ER, which aims to 1) restore protein folding, through increasing ER chaperones and 2) decrease general protein translation and thus reducing the accumulation of unfolded proteins (Ron and Walter, 2007). Weber and colleagues (2004) demonstrated that PPARγ ligands induced ER stress activation through the expression of genes regulated by the UPR, such as Hsp70 and GRP78/BIP. The authors also found that ER stress activation protected β-cells from harmfull effects of cytokines signalling suggesting a new protective potential for UPR activation (Weber et al., 2004). However, when the adaptation fails and/or under extreme/prolonged ER stress, the UPR prosurvival signals become proapoptotic (Kim et al., 2006a; Szegezdi et al., 2006; Xu et al., 2005). Taking this finds into account, we decide to analyse the effects of L7G on the hepatic GRP78/BIP protein expression and the results demonstrated that, this phytochemical, up-regulated this chaperone levels, without inducing proapoptotic signals through JNK or caspase-3 activation. Moreover, L7G treatment not only failed to induce the hepatic protein levels of FAS or ACC, but also showed a tendency to decrease SREBP-1 mRNA levels, which may indicate a potential role for this phytochemical preventing SREBP-1 proteolytic cleavage and/or gene expression. Further research must be conducted in order to better clarify this potential role of L7G on SREBP-1 regulation although, the involvement of PPARα and/or GRP78/BIP activation should also be considered.

SREBP-2 mRNA levels were not significantly modified by dietary supplementation with L7G. However, the expression levels of SREBP-2 target genes, LDLR and HMGCR, disclose an interesting pattern: while the former was not significantly modified under L7G treatment, the later may reflect a potential role for L7G reduce cholesterol endogenous synthesis. In fact, L7G significantly inhibited, in a dose-dependent way, the *in vitro* activity of the HMGCR, although in a less extent than

pravastin (statin). These data may indicate a distinct transcriptional regulation for both LDLR and HMGCR genes that requires further investigation.

The study reported herein added new insights on the lipid-lowering potential of L7G, a phytochemical highly abundant in several fruits and vegetables. Taken together, these evidences may assist our belief that L7G may activate or act as potential PPARα agonist and stimulate the expression of genes responsible for the previous lipid-lowering effects. Additionally, L7G may exerts its effects by blocking the SREBP-1 processing and/or expression and thus, inhibiting the consequent lipogenic action of this transcription factor. Studies are undergoing to elucidate the unanswered questions emerged with this work and to compare the effects of L7G here reported with the aglycone luteolin.

## Acknowledgements

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# CHAPTER 5

## *In vitro regulation of intestinal sodium-glucose cotransporter 1 (SGLT1) in response to dietary factors*

## 5.1. Chapter overview

In a preceding study performed in our laboratory it was observed that SFT and particularly RA, significantly repressed the diet-induced boost of SGLT1 expression at the rat intestinal BBM[1]. However, no effects on the BLM levels of $Na^+/K^+$-ATPase and GLUT2 in whole cell homogenates were detected[1]. Based on these evidences and conscious that little is known about the molecular mechanisms of SGLT1 regulation and translocation from intracellular pools to the BBM in the enterocytes, we performed the work presented in this chapter.

Butyrate (NaBu)-induced differentiated HT-29 cells and spontaneously differentiated Caco-2 cells were used, in order to choose the best *in vitro* model for study the intestinal cellular adaptations in response to glucose concentration and phytochemicals, like RA. The cellular growth and differentiation status of both cell lines seemed to be affected differently by glucose. NaBu increased the total expression of SGLT1 and GLUT2 proteins in HT-29 cells, especially grown under high glucose levels. A subcellular fractionation procedure revealed that NaBu increased the expression of SGLT1 at the BBM, whereas GLUT2 expression increased in the remaining membrane fraction. Nonetheless, RA did not inhibit the glucose-associated increase of BBM SGLT1 expression as demonstrated *in vivo*, under dietary carbohydrate manipulation. In addition, SGLT1 protein location and expression in HT-29 cells did not seem to be modified by insulin. In Caco-2 cells, both SGLT1 and GLUT2 transporters seem to have the same intracellular location as observed in HT-29 cells thought, no changes in their protein expression was observed after glucose and/or insulin exposure. Thus, glucose in medium was able to induce SGLT1 levels in the BBM of NaBu-differentiated HT-29 cells, but RA was unable to modulate it.

Consequently and despite the value of HT-29 and Caco-2 cells for drug absorption studies, further work needs to be developed to clarify the regulation of SGLT1 by dietary constituents by using alternative experimental models and/or approaches than the ones already used.

[1] Azevedo MF, Lima CF, Fernandes-Ferreira M, Almeida MJ, Wilson JM and Pereira-Wilson C (2011) Rosmarinic acid, major phenolic constituent of Greek sage herbal tea, modulates rat intestinal SGLT1 levels with effects on blood glucose. *Mol Nutr Food Res* **55 Suppl 1**:S15-25.

## 5.2. Manuscript 3

This chapter comprises the following manuscript:

**Carla M Sá**, Cristovao F. Lima and Cristina Pereira-Wilson (**2012**). Glucose induces the levels of SGLT1 in the brush-border membrane of butyrate-induced enterocyte differentiation from HT-29 cells (*in preparation*).

# Glucose induces the expression of SGLT1 in the brush-border membrane of butyrate-induced enterocyte differentiation from HT-29 cells

Carla M. Sá[1], Cristovao F. Lima[2] and Cristina Pereira-Wilson[1]

[1] *CBMA - Centre of Molecular and Environmental Biology/ Department of Biology, University of Minho, 4710-057 Braga, Portugal.*

[2] *CITAB - Centre for the Research and Technology of Agro-Environmental and Biological Sciences/Department of Biology, University of Minho, 4710-057 Braga, Portugal.*

## Abstract

Type 2 diabetes mellitus (T2DM) is assuming global alarming proportions. The need of novel interventions for controlling and preventing diabetes is rising, renewing the interest to search for new active compounds. In recent *in vivo* experiments, we confirmed the glucose lowering properties attributed by popular medicine to *Salvia fruticosa* plants (family Laminaceae) and its main phenolic compound rosmarinic acid (RA). In the present study, we aimed to establish an *in vitro* model to study mechanisms of regulation of sodium-glucose cotransporter 1 (SGLT1) response to dietary constituents, such as glucose, phytochemicals and insulin. For this purpose differentiated Caco-2 cells and butyrate (NaBu)-induced differentiated HT-29 cells were used, since they are often considered good *in vitro* models of the intestinal absorptive cells. Glucose seems to affect differently the cellular growth and differentiation patterns of both cell lines. In HT-29 cells, butyrate increased the levels of SGLT1 and GLUT2 transporters, to a higher extent in the presence of high concentration of glucose in the medium. By subcellular fractionation, the increase of SGLT1 and GLUT2 by NaBu was at the apical (BBM) and on the remaining membrane fraction, respectively. Contrarily to previous *in vivo* experiments, RA did not prevent the increase of SGLT1 levels at BBM fraction. Insulin did not seem to affect SGLT1 protein location and expression in HT-29 cells. The same pattern of intracellular distribution of SGLT1 and GLUT2 was observed in Caco-2 cells, however, their protein expression was not affected by glucose nor insulin. Notwithstanding the general acceptance of Caco-2 and HT-29 cells for drug absorption studies, further work must be conducted in order to elucidate the previous observed RA-mediated effects on BBM SGLT1 expression.

**Key words:** Type 2 diabetes mellitus - sodium butyrate - SGLT1 - rosmarinic acid - glucose.

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## 1. Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder strongly related to sedentary lifestyle and obesity, which is caused by defects in both insulin production and/or action (Curtis and Wilson, 2005; Hanhineva et al., 2010). The impact of diabetes and its associated complications are achieving massive medical and socioeconomic proportions owing to the economic burden but also to the lost of productivity as a result of diabetes-related premature morbidity and mortality (Stumvoll et al., 2005). Therefore, the search and implementation of new strategies to prevent or delay T2DM onset becomes pivotal.

The maintenance of glucose homeostasis is of great physiological importance and also dependent on intestinal mechanisms. At the intestinal level, luminal glucose derived from digestible carbohydrates is absorbed by the sodium-glucose cotransporter 1 (SGLT1), a high affinity glucose transporter that drives sugar transport across the brush-border membrane (BBM) of the enterocyte, using the electrochemical gradient of sodium (Wright et al., 2007). Sugar accumulation within the enterocytes is transported into the blood by facilitated diffusion through GLUT2, which is expressed at the basolateral membrane (BLM) (Wright et al., 2007). Intestinal sugar absorption is modulated by dietary carbohydrates since increasing digestible carbohydrates leads to a boost in SGLT1 and GLUT2 expression (Cheeseman and Harley, 1991). This increase in BBM SGLT1 is also observed in diabetes, where higher levels of intestinal glucose transporters, particularly SGLT1 at BBM, leads to an increase in intestinal glucose absorption and contributes negatively to increase blood glucose (Diamond et al., 1984; Dyer et al., 2002; Ferraris, 2001).

Several medicinal plants have been reported to possess antidiabetic properties (Alarcon-Aguilar et al., 2002; Lima et al., 2006). Among them, species from the genus *Salvia* (family Laminaceae), including *S. officinalis* and *S. fruticosa*, have been empirically used since ancient times to treat diabetes. This popular belief has been corroborated by our previous experiments: 1) fourteen days treatment *S. officinalis* extract (SOT) enhances the hepatocyte responsiveness to insulin by decreasing hepatic

gluconeogenesis (Lima et al., 2006); 2) a pilot trial with non-diabetic female volunteers showed that SOT improves lipid profile, lymphocyte Hsp70 protein expression and SOD and CAT antioxidant activities (Sa et al., 2009), 3) streptozotocin-induced diabetic rats showed that *S. fruticosa* extract (SFT) reduces the diabetes-associated raise of the SGLT1 protein expression in BBM without affecting GLUT2 and $Na^+/K^+$-ATPase protein expression (Azevedo et al., 2011) and finally 4) diet-induced increase on BBM SGLT1 levels was remarkably inhibited by SFT drinking (Azevedo et al., 2011).

Both *Salvia* species are rich sources of active compounds that confer its claimed medicinal and aromatic properties. Rosmarinic acid (RA) is the major phenolic compound present in SFT and SOT where it comprises around 72% and 70% of all phenolic compounds, respectively (Azevedo, 2008; Lima, 2006; Lima et al., 2007). Among the multiple biological activities described for RA (Petersen and Simmonds, 2003), we have recently demonstrated that, this compound, seems to be an active constituent of *Salvia* species since it repressed the *in vivo* carbohydrate-induced adaptive increase of BBM SGLT1, an effect accompanied by a reduction in plasma glucose levels (Azevedo et al., 2011).

Cellular *in vitro* approaches are usually used to explore the molecular mechanisms of phytochemicals. Regarding the human carbohydrate intestinal absorption, Caco-2 and HT-29 human colonocyte cell lines are often considered good *in vitro* models due to their ability to undergo differentiation into polarized epithelial cells in culture, and expressing distinctive brush-border enterocyte markers such as alkaline phosphatase, sucrase-isomaltase and aminopepetidase (Huet et al., 1987; Simon-Assmann et al., 2007; Thomson and Wild, 1995). Babia and colleagues (1989) demonstrated that insulin stimulates glucose uptake and lactate production in a dose-dependent way in HT-29 cells cultured in high glucose medium. In this condition, the authors also observed an increase in the levels of fructose 2,6-biphosphate which was followed by an increase in the glycolytic rate of these cells (Babia et al., 1989). Furthermore, it has been reported that, in Caco-2 cells, SGLT1 resides in microtubule-associated vesicles and responds rapidly and efficiently to mechanisms of vesicle trafficking (Khoursandi et al., 2004; Kipp et al., 2003). However, Khoursandi and colleagues (2004) suggested that additional mechanisms for SGLT1 regulation may coexist since they found that the subcellular distribution of SGLT1 was not altered in Caco-2 cells after exposure to either free or high D-glucose medium.

The present work aims to establish an *in vitro* model to study the mechanism of regulation of glucose transporters, particularly SGLT1 in response to dietary constituents, such as glucose and also insulin, to then test *in vitro* the effects of RA on SGLT1 expression. To study the mechanisms that regulate SGLT1 translocation from intracellular pools to the BBM, a subcellular fractionation approach was performed in sodium butyrate (NaBu)-induced differentiated HT-29 cells and spontaneously differentiated Caco-2 cells.

## 2. Materials and Methods

### *2.1 Reagents and antibodies*

Rosmarinic acid (RA), sodium butyrate (NaBu), RPMI medium, DMEM medium, antibiotic/antimicotic solution, Trypsin-EDTA solution (0.25%), MEM non-essential aminoacids solution (100x), insulin, D-mannitol, D-glucose, Bradford reagent and anti-β-Actin antibody were acquired from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Lonza (Verviers, Belgium). PNGase F was purchased from New England BioLabs Inc. (Herts, UK). Antibodies against p21, p27 and the secondary antibody HRP anti-mouse were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Two different antibodies raised against residues 603-623 or to amino acids 402-422 of human SGLT1 (SGLT1a and SGLT1b, respectively) were bought from Abcam (Cambridge, UK). GLUT2 antibody was purchased from Chemicon/Millipore (Billerica, MA, USA). Phosphorylated Akt and total Akt were acquired from Cell signalling (Danvers, MA, USA). The secondary antibody HRP anti-rabbit was obtained from Cell Signaling (Danvers, MA, USA). All other chemicals or solvents were of analytical grade.

### *2.2. Cell culture maintenance*

Caco-2 and HT-29 human colon carcinoma cells were kindly provided by Dr. Karsten Kristiansen (University of Copenhagen, Denmark). The cells were routinely maintained at 37ºC in a humidified 5% $CO_2$ atmosphere and grown in DMEM supplemented with 10% FBS, 10 mM Hepes and 100 IU/ml antibiotic/antimicotic

solution. Caco-2 medium was supplemented with 1% non-essential aminoacids solution. The medium was renewed every 2 days.

*2.3. Differentiation to enterocytes*

Differentiation of Caco-2 and HT-29 cells to enterocytes were done by elsewhere published protocols (Chang et al., 2007; Engle et al., 1998; Hodin et al., 1996; Wang et al., 2001). In brief, Caco-2 cells were differentiated by growing cells at confluency continuously for at least 15 days. For HT-29 cell line, after cells reached confluence, differentiation was induced with 5 mM of NaBu for 2 days. In order to study the metabolic response of enterocytes to glucose using differentiated HT-29 cells, undifferentiated cells were routinely grown in RPMI medium containing 5.56 mM glucose (normoglycaemic medium) or 25 mM (hyperglycaemic medium) to resemble a physiological and a diabetic condition, respectively. The 5.56 mM glucose medium was adjusted with D-mannitol to maintain osmolarity of the hyperglycaemic condition.

*2.4. Cell counting*

During the exponential phase of cell growth, cell counting was performed in cells growing in the two different glucose concentration media. For that, cells were washed with PBS, harvested following trypsin-EDTA treatment, resuspended in culture media and then counted using a hemocytometer. Counts were performed after 48 and 72 h in culture. For the 72 h time point, culture medium was renewed after 48 h of culture.

*2.5. Alkaline phosphatase activity*

Alkaline phosphatase (ALP) activity was measured by using disodium *p*-nitrophenyl phosphate as substrate as elsewhere (Pekarthy et al., 1972; Wright, 1977). In brief, cells were lysed in ice-cold 0.25% Triton X-100 with a brief sonication, and homogenate centrifuged at 10,000 x g, at 4ºC and during 10 min. The ALP activity of the cells was assessed spectrophotometrically following the formation of *p*-nitrophenol at 420 nm along the time on a plate reader (Spectra Max 340 pc, Molecular Devices, Sunnyvale, CA, USA). Protein was quantified by the Bradford method (Sigma-Aldrich). Enzyme activity was expressed in μmol *p*-nitrophenyl phosphate/min/mg protein.

*2.6. Total protein extraction*

To prepare total cell homogenates, HT-29 cells were washed with PBS and lysed in ice-cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA), supplemented with 20 mM NaF, 20 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl flouride (PMSF) and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified using Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) with BSA as a protein standard. Equal amount of protein cell lysates (20 μg) were separated by SDS-PAGE.

*2.7. Deglycosylation assays*

N-linked deglycosilation of SGLT1 in Caco-2 and HT-29 cells was accomplished by treating the total cell homogenates with peptide-$N^4$-(Nacetyl-β-glucosaminyl) asparagine amidase F, also known as PNGase F, from *Flavobacterium meningosepticum*, following manufacturer's specifications. Cell lysates samples (corresponding to 20-30 μg protein) were solubilised, boiled for 5 min at 100ºC and incubated for 2 h at 37ºC with 2,500 units of PNGase. Equal amount (20 μg) of these samples was separated by SDS-PAGE. BBM rat protein sample was used as a positive control.

*2.8. Subcellular fractionation*

BBMs were isolated from the remaining membrane fraction (basolateral plasma membrane and internal membrane fraction) by $MgCl_2$-induced membrane precipitation as elsewhere (Navas et al., 1989). In brief, cells grown in 10 cm petri-dishes until differentiation were washed and scraped with ice-cold PBS and left for 10 min in ice-cold homogenisation buffer A (10 mM Hepes, pH 7.4, 15 mM KCl and 15 mM $MgCl_2$, supplemented with 1 mM PMSF and 1 mM DTT) and subsequently homogenised with 40 strokes of a Dounce-homogeniser using a tight-fitting pestle. Nuclei and entire cells were pelleted by centrifugation at 700 x g for 10 min at 4ºC. Then, supernatant (designated as *whole cell homogenate fraction* - WCHF), was centrifuged at 10,000 x g for 30 min at 4ºC to collect the supernatant (designated as *brush-border membrane plus cytosolic fraction* – BBMF + CF) and the pellet. This resultant pellet was then resuspended in 0.25% Triton X-100 and was designated as *basolateral and internal*

*membrane fraction* – BIMF. All fractions were subjected to SDS-PAGE after protein quantification using Bradford reagent. The loading of protein amount for each subcellular fraction was: WCHF (30 μg); BBMF + CF (20 μg) and BIMF (20 μg). This procedure outline is shown on **Figure 1**.

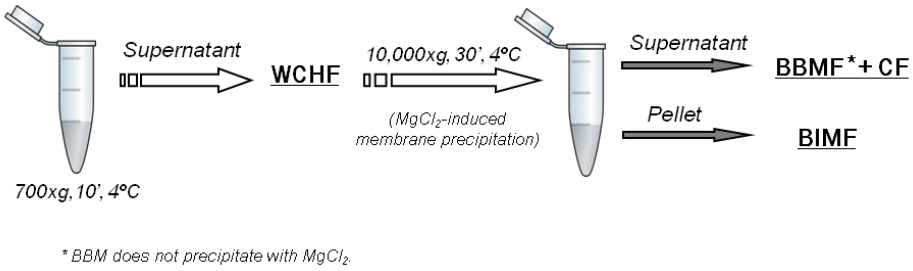


**Figure 1.** Schematic representation of the outline used in the subcellular fractionation procedure. The cellular fractions used are indicated in the scheme: w*hole cell homogenate fraction* (WCHF); *brush-border membrane and cytosolic fraction* (BBMF + CF); *basolateral and internal membrane fraction* (BIMF).

### *2.9. Western blotting*

Proteins were separated by SDS-PAGE and then transblotted onto to Hybond-P polyvinylidene difluoride membrane (GE Healthcare, UK). Membranes were then blocked in 5% (w/v) non-fat dry milk in TPBS (0.05% (v/v) Tween 20 in PBS, pH 7.4). Blotted membranes were probed with primary antibody and then detected by chemiluminescence using appropriate secondary antibodies and the reactive bands acquired with a ChemiDoc XRS (Bio-Rad) imaging densitometer. Band area intensity was quantified using the Quantity One image analysis software from Bio-Rad. β-Actin was used as a loading control.

### *2.10. Statistical Analysis*

Data are presented as means ± SEM of at least 3 independent experiments. For statistical analysis, the different parameters were analysed by the Student's *t*-test, by one-way ANOVA or by two-way ANOVA (both followed by the Bonferroni test), as appropriate, using GraphPad Prism 4.0 software (San Diego, CA, USA). *P* values ≤ 0.05 were considered statistically significant.

## 3. Results

### *3.1. Glucose affects differently the cellular growth and differentiation status of both Caco-2 and HT-29 cells*

To study the effect of glucose concentration on SGLT1 maturation in BBM of differentiated Caco-2 and HT-29 cells, first we investigated the effects of glucose on cell growth and differentiation. As observed in **Figure 2**, cell number increased significantly in both cell lines until 72 h ($P \leq 0.001$), with no significant changes between the lower and higher concentration of glucose in Caco-2 cells (**Figure 2B**). However, glucose concentration in culture medium affected differentially HT-29 cells (**Figure 2A**). Even changing the medium at 48 h, growth of HT-29 cells decreased significantly at 72 h in 5.56 mM glucose medium when compared with that of cells grown in 25 mM glucose medium.

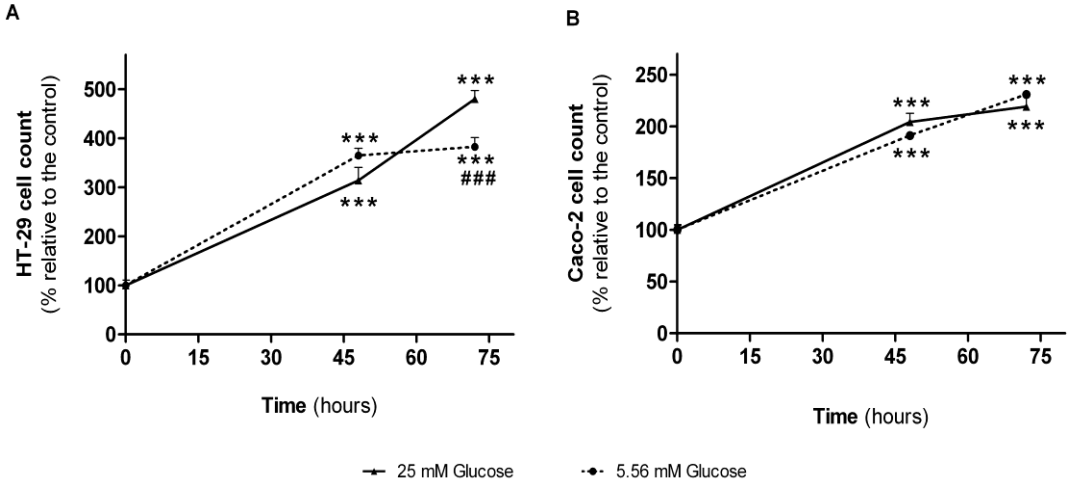


**Figure 2.** Cell growth curve of HT-29 [**A**] and Caco-2 [**B**] cells cultured in RPMI medium containing different glucose concentrations (5.56 and 25 mM). Values are means ± SEM of at least three independent experiments. $***P \leq 0.001$ when compared with the respective control by the one-way ANOVA. $^{\#}P \leq 0.05$ and $^{\#\#\#}P \leq 0.001$ when compared the same condition in 25 mM glucose medium by the two-way ANOVA.

To follow cell differentiation, the activity of ALP was used. As expected, after addition of NaBu, the activity of ALP increased in HT-29 cells, however, this increase was much more significant in cells grown in 25 mM glucose medium (**Figure 3A**). Similar to HT-29, Caco-2 cells presented a higher activity of ALP in 25 mM glucose medium compared to that of 5.56 mM medium (**Figure 3B**). According to the ALP results, Caco-2 cells spontaneously differentiated after 21 days in culture in 25mM glucose, whereas for 5.56 mM glucose the maximal differentiation status was already

reached after 14 days, having into account the plateau observed. In the following experiments with Caco-2 cells, 15 days of cell differentiation was used for both culture media.

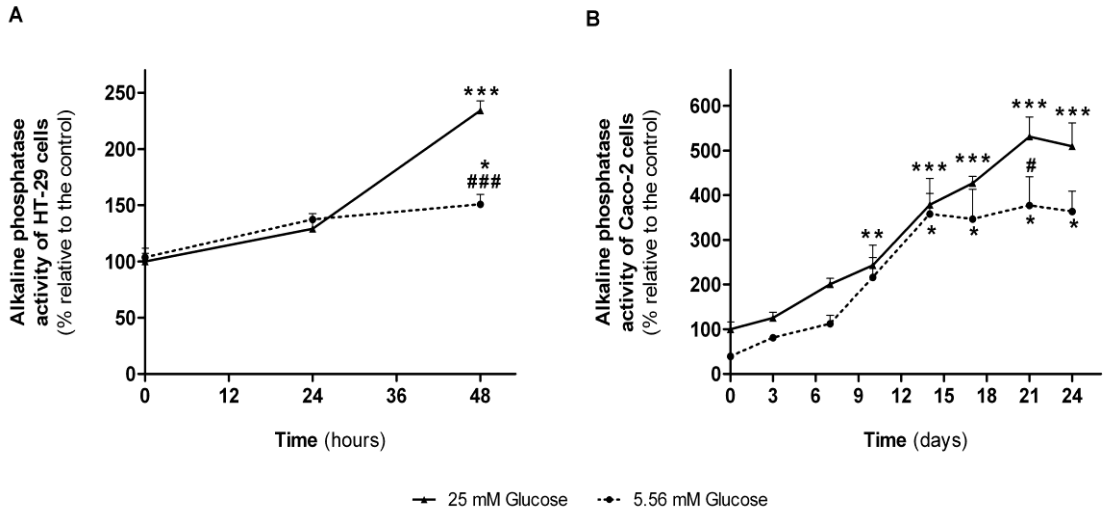


**Figure 3.** Alkaline phosphatase activity of HT-29 [**A**] and Caco-2 [**B**] cells cultured in 5.56 and 25 mM glucose media. In HT-29 cells, after adhesion and cell confluency, differentiation was induced by the incubation of cells with 5 mM NaBu for 24 and 48 h. Before NaBu treatment, a sample of cells was taken to serve as control (time 0 h). In Caco-2 cells, spontaneous differentiation was induced by growing the culture at confluency during 24 days. Values are means ± SEM, of at least three independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ when compared with the respective control by the one-way ANOVA. Two-way ANOVA indicates differences between the same condition in different glucose media ($^{\#}P \leq 0.05$; $^{\#\#\#}P \leq 0.001$).

To confirm differentiated status of HT-29 cells just after 48 h of NaBu addition, the expression levels of cell cycle inhibitor markers p21 and p27 proteins were followed by western blot. As shown in **Figure 4 A**, **B** and **E**, the levels of p21 and p27 increased significantly in both glucose media, with a tendency of higher expression in the 25 mM glucose medium. Therefore, based on ALP and western blot results, treatment of HT-29 cells with NaBu for 48 h was used to differentiate these cells in the following experiments.

### *3.2. Butyrate induces the levels of intestinal glucose transporters SGLT1 and GLUT2 in total homogenates of HT-29 cells*

The effect of NaBu-induced differentiation of HT-29 cells cultured in two glucose concentrations in the levels of intestinal glucose transporters were studied by western blot. As shown in **Figure 4E**, HT-29 cells exhibit two immunoreactive bands when

membranes were probed against both SGLT1 antibodies. The same result was obtained whether the SGLT1a (raised against residues 603-662) or SGLT1b (raised against the residues 402-422) antibodies were used. The upper band has a molecular weight of approximately 80 kDa (the same size than the one found for Caco-2 cells) and the lower band has approximately 75 kDa (**Figure 4E**). Butyrate treatment particularly increased the levels of the 80 kDa SGLT1 band (**Figure 4C** and **E**), but not the 75kDa band (**Table 1**).

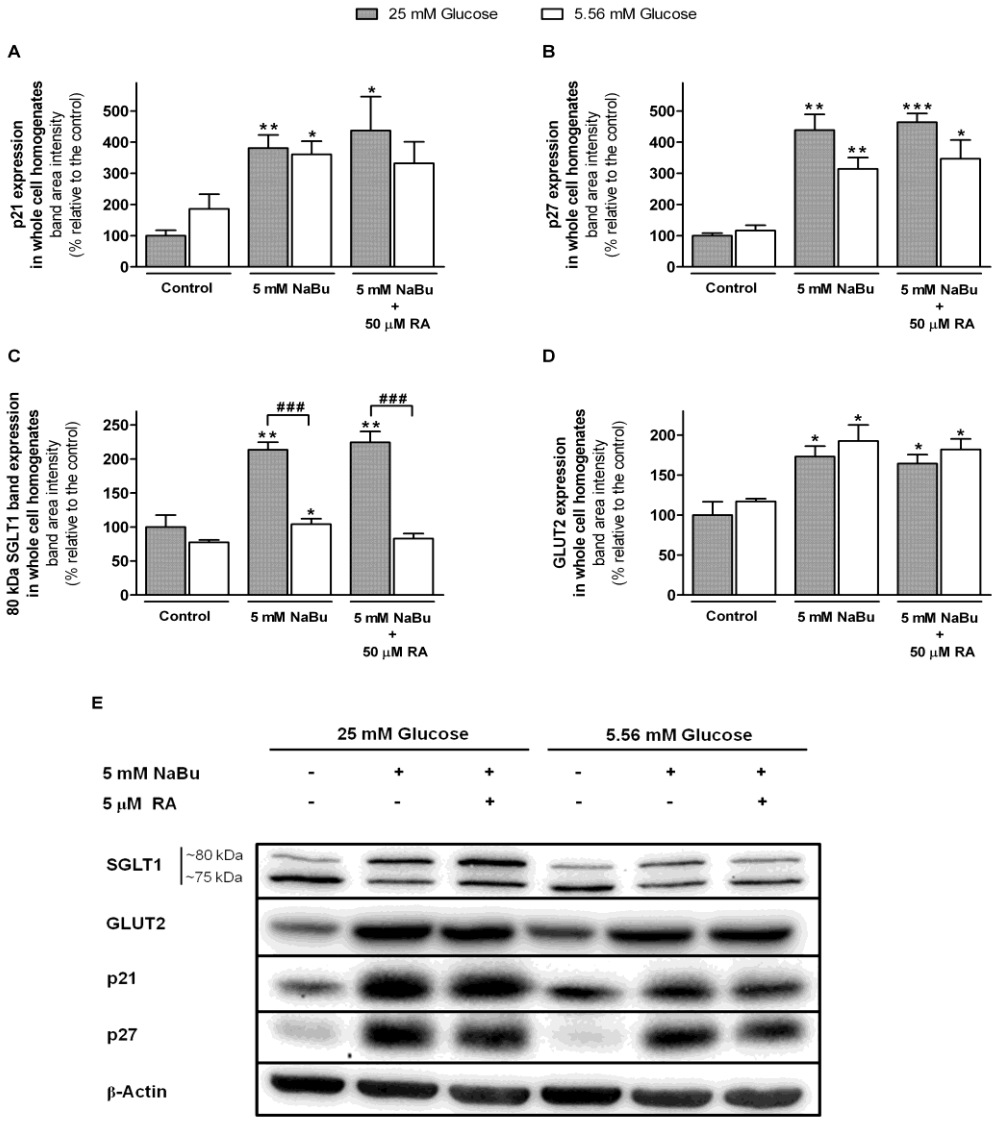


**Figure 4.** Effect of 5 mM NaBu and 50 μM RA for 48 h in the levels of differentiation markers p21 [**A**] and p27 [**B**], as well as the glucose transporters SGLT1 [**C**] and GLUT2 [**D**], in whole cell homogenates of HT-29, as assessed by western blot. White and grey bars represent cells grown in culture medium containing 5.56 mM and 25 mM glucose, respectively. SGLT1 levels (**C**) are from the 80 kDa band (for other bands see **Table 1**). Values are means ± SEM of three independent experiments. $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.001$ when compared with the respective control; $^{\#\#\#}P \leq 0.001$when compared with each other by the Student's *t*-test. If nothing specified, there are no differences between the same condition in different glucose media. Representative blots and corresponding loading control (β-Actin) are present in [**E**].

Nevertheless, this effect is still observed if the sum of both SGLT1 bands (80 kDa + 75 kDa band) is used (**Table 1**). Interestingly, mimetizing the phenomenon *in vivo*, such as the effect of diabetes or dietary carbohydrate manipulation (Azevedo et al., 2011), total levels of SGLT1 in differentiated cells increased more significantly in the 25 mM glucose medium (**Figure 4C** and **E**, **Table 1**). To verify if the two bands pattern could disclose a process of protein maturation, since SGLT1 is usually glycosylated *in vivo*, cell lysates were treated with PNGase F. As shown in **Figure 5A** and **5B**, after PNGase F treatment, the original bands did not disappear and no new band with lower molecular weight appeared, indicating that SGLT1 are deglycosylated *in vitro* in both HT-29 and Caco-2 cells. As expected, PNGase F treatment decreased the molecular weight of SGLT1 protein bands from rat BBM protein fraction from approximately 87 to 70 kDa (**Figure 5B**). The appearance of a band at 40 kDa for Caco-2 cells was observed (**Figure 5**), probably due to unspecific imunocrossreactivity.

**Table 1.** Effect of 5 mM NaBu and 50 µM RA for 48 h in the protein levels of SGLT1 in whole cell homogenates of HT-29, as assessed by western blot. Values are in % relative to 80 kDa SGLT1 band from untreated HT-29 cells grown in 25 mM glucose medium (see **Figure 4C**).

| | **75 kDa** ***(Lower band)*** | | **Band sum** ***(80 + 75 kDa band)*** | |
|---|---|---|---|---|
| | **25 mM** | **5.56 mM** | **25 mM** | **5.56 mM** |
| **Control** | 165.4 ± 49.5 | 124.8 ± 31.5 | 265.4 ± 60.1 | 202.2 ± 27.5 |
| **5 mM NaBu** | 147.6 ± 30.2 | 100.6 ± 11.1 $^{(\#)}$ | 360.9 ± 43.0 $^{(*)}$ | 204.7 ± 15.2 $^{(\#\#\#)}$ |
| **5 mM NaBu + 50 µM RA** | 149.4 ± 26.0 | 105.6 ± 32.6 | 373.9 ± 34.5 $^{(*)}$ | 188.6 ± 36.1 $^{(\#\#\#)}$ |

Values are means ± SEM of three independent experiments. $^{*}P \leq 0.05$ when compared to the respective control; $^{\#}P \leq 0.05$; $^{\#\#\#}P \leq 0.001$ when compared the same condition in 25 mM glucose medium by the Student's *t*-test. If nothing specified, there are no differences between the same condition in different glucose media.

A significant increase in GLUT2 levels was also observed after NaBu treatment in HT-29 cells grown in both glucose concentrations (**Figure 4D** and **E**). Treatment with NaBu seems also to interfere with cellular cytoskeleton structure/composition of HT-29 cells, since β-Actin levels decreased after 48 h, as shown in **Figure 4E**. For this reason, β-Actin was not used as a loading control. Like in *in vivo* results (Azevedo et al., 2011), RA did not change total expression of SGLT1 and GLUT2 proteins, when compared with NaBu alone (**Figure 4C**, **D** and **E**).

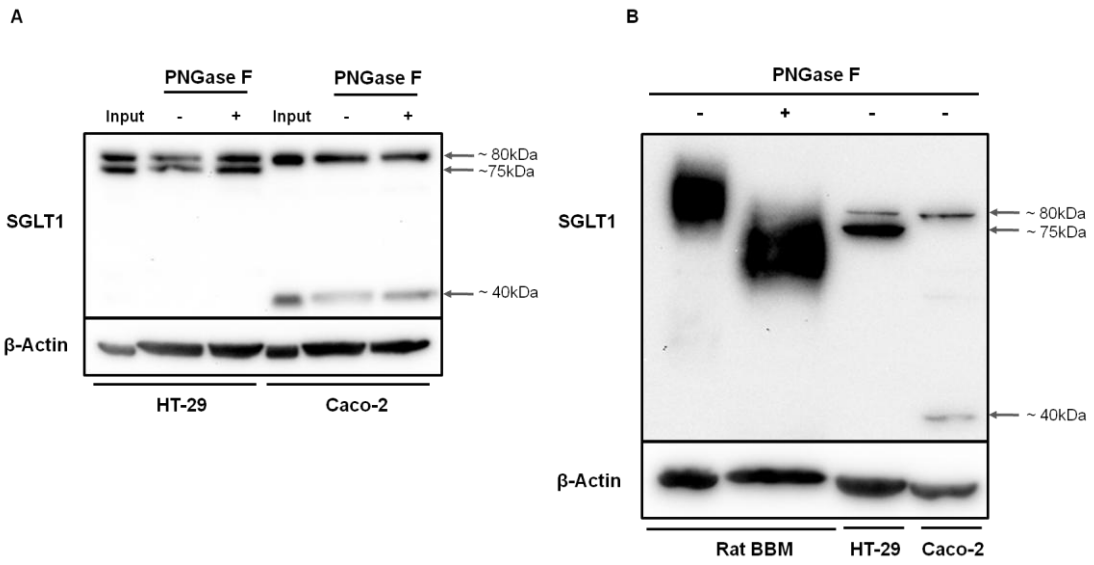


**Figure 5.** Effect of treatment of cell lysates from HT-29 and Caco-2 cells with PNGase F enzyme in the molecular weight of imunoreactive bands detected by SGLT1 antibody. The blots were probed with different SGLT1 antibodies, SGLT1a [**A**] and SGLT1b [**B**], raised against different amino acids residues. BBM from a rat jejunum sample was used as a control for the deglycosilation reaction [**B**]. Input samples refer to the corresponding cell lysate that were not processed by the PNGase F method. β-Actin was used as a loading control.

### *3.3 Glucose affects the expression of SGLT1 on BBM fractions of differentiated HT-29 cells*

To clarify the intracellular distribution and levels of glucose transporters SGLT1 and GLUT2 in response to glucose and RA, a subcellular fractionation procedure was carried out in HT-29 cells. As shown in **Figure 6A** and **6D**, HT-29 cells grown in low glucose medium presented most of SGLT1 in the BBM fraction, before or after NaBu treatment, probably due to a higher glucose transport capacity in the presence of limited amounts of glucose in the medium. On the other hand, in cells grown in high glucose medium, a significant amount of SGLT1 was detected in the BIM fraction (**Figure 6A** and **6C**), probably due to SGLT1 association with vesicles. However, when cells were differentiated with NaBu, a significant increase of SGLT1 levels (upper band) in the BBM fraction was observed with the corresponding decrease in the remaining membrane fraction (BIMF). The levels of the lower SGLT1 band (75 kDa) did not significantly change in the BBMF after NaBu treatment, but decreased remarkably in the BIMF in cells grown in high glucose medium (**Table 2**, **Figure 6C**).

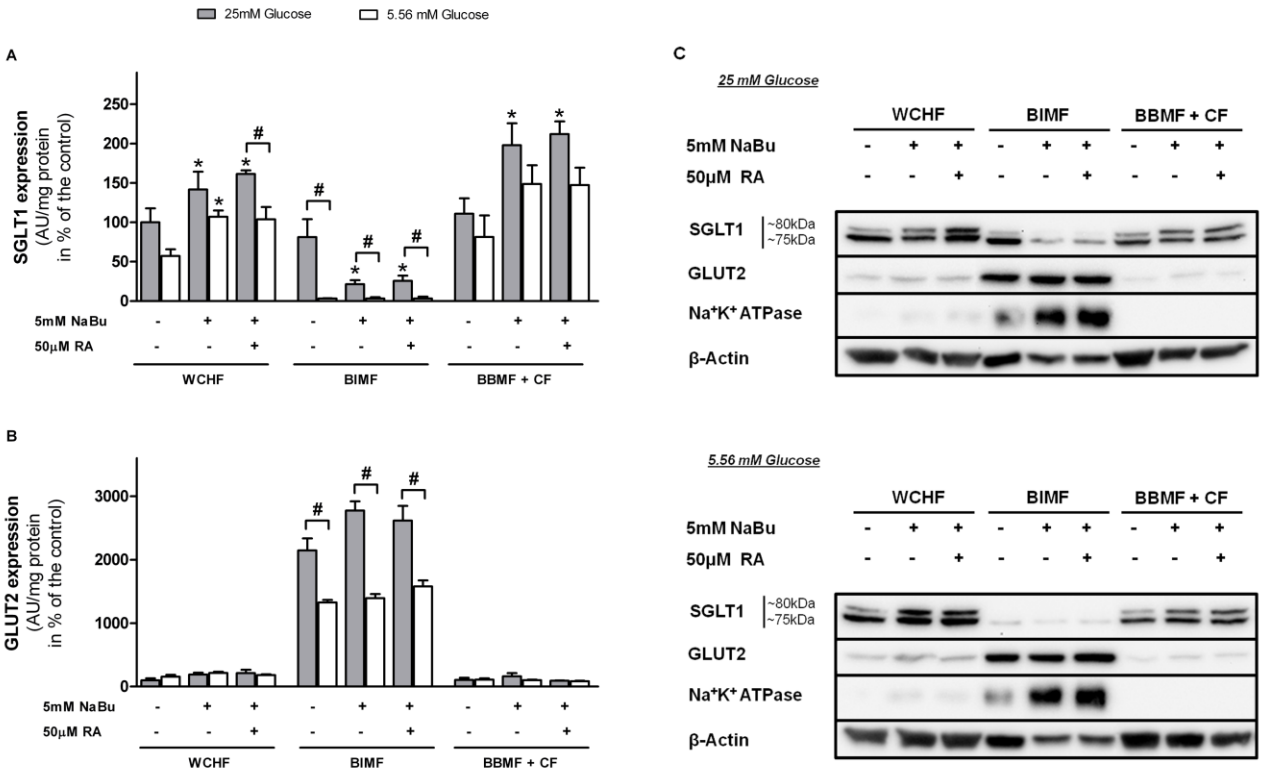


**Figure 6.** Effect of 5 mM NaBu and 50 μM RA for 48 h in the levels of SGLT1 [**A**] and GLUT2 [**B**] glucose transporters in different subcelluar fractions (**Fig. 1**) of HT-29 cells grown in culture medium containing 5.56 mM (white bars) or 25 mM (grey bars) glucose, as assessed by western blot. SGLT1 levels [**A**] are from the 80 kDa band (for other bands see **Table 2**). Values are means ± SEM of three independent experiments. $^{*}P \leq 0.05$ when compared with the respective control; $^{\#}P \leq 0.05$ when compared with each other by the Student's *t*-test. If nothing specified, there are no differences between the same condition in different glucose media. Representative blots and corresponding loading control (β-Actin) are present in [**C**] and [**D**] for cells grown in culture medium containing 25 mM and 5.56 mM glucose, respectively.

In both glucose media, GLUT2 expression was remarkably higher in the BIMF as compared with other fractions (**Figure 6B**, **6C** and **6D**), and may represent its expression in the basolateral plasma membrane. In fact, the protein levels of the basolateral plasma membrane marker $Na^{+}/K^{+}$-ATPase was only detected in the BIMF (**Figure 6C** and **6D**). Interestingly, its expression levels were increased with butyrate in cells grown in both glucose concentrations. β-Actin was not used as a loading control since its expression was affected by NaBu treatment (**Figure 6C**). Therefore, values were normalized according to the protein amount loaded for each subcellular fraction.

**Table 2.** Effect of 5 mM NaBu and 50 µM RA for 48 h in the levels of SGLT1 in different subcelluar fractions (**Figure 1**) of HT-29 cells grown in culture medium containing 5.56 mM or 25 mM glucose, as assessed by western blot. Values are in % relative to 80 kDa SGLT1 band from untreated HT-29 cells grown in 25 mM glucose medium (see **Figure 6A**).

| | **25 mM** | | | | | | **5.56 mM** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **75 kDa** *(Lower band)* | | | **Band sum** *(80 + 75 kDa band)* | | | **75 kDa** *(Lower band)* | | | **Band sum** *(80 + 75 kDa band)* | | |
| | *WFCH* | *BIMF* | *BBMF + CF* | *WFCH* | *BIMF* | *BBMF + CF* | *WFCH* | *BIMF* | *BBMF + CF* | *WFCH* | *BIMF* | *BBMF + CF* |
| **Control** | 216.1 ±44.0 | 295.1 ±98.4 | 252.0 ±31.2 | 316.1 ±32.4 | 376.4 ±133.7 | 362.8 ±29.9 | 143.4 ±18.7 | 45.8 ±23.3 (#) | 204.9 ±78.5 | 200.7 ±31.0 (#) | 49.1 ±23.9 (#) | 286.3 ±123.3 |
| **5 mM NaBu** | 176.8 ±8.7 | 93.9 ±29.7 (*) | 232.2 ±85.5 | 318.5 ±47.7 | 115.4 ±37.6 (*) | 430.2 ±131.1 | 155.4 ±1.2 (#) | 32.1 ±19.5 | 205.5 ±50.4 | 262.3 ±12.9 (*) | 35.5 ±21.6 (#) | 354.3 ±80.6 |
| **5 mM NaBu + 50 µM RA** | 217.7 ±46.2 | 88.5 ±41.3 (*) | 232.2 ±45.5 | 379.2 ±47.2 | 114.1 ±53.1 (*) | 444.2 ±70.5 | 153.1 ±17.5 | 29.4 ±18.8 | 195.6 ±54.8 | 256.8 ±18.2 (#) | 32.8 ±22.5 (#) | 342.8 ±85.9 |

Values are means ± SEM of three independent experiments. *$P \leq 0.05$ when compared with the respective control; #$P \leq 0.05$ when compared to the same condition in 25 mM glucose medium by the Student's *t*-test. If nothing specified, there are no differences between the same condition in different glucose media.

With the fractionating procedure, some different results were observed when comparing WCHF (**Figure 6**) with total expression on **Figure 4**. That may be due to the differences in the homogenization/lysis buffer, which lacks detergent in the fractionation procedure.

Contrarily to the previous *in vivo* experiments (Azevedo et al., 2011), RA did not inhibited the glucose-induced increase of SGLT1 levels in the BBM of differentiated HT-29 cells (**Figure 6A-D**).

### *3.4. Insulin did not affect SGLT1 expression in HT-29 cells*

In order to investigate the possible effect of insulin on SGLT1 regulation in differentiated HT-29 cells grown in 5.56 mM or 25 mM glucose, cells were pre-treated with NaBu and/or RA for 48 h and then incubated with 100 nM insulin for 30 min. The

incubation time of insulin was chosen based on activation of phosphorylated Akt, an indicator of insulin signalling pathway activation (**Figure 7**).

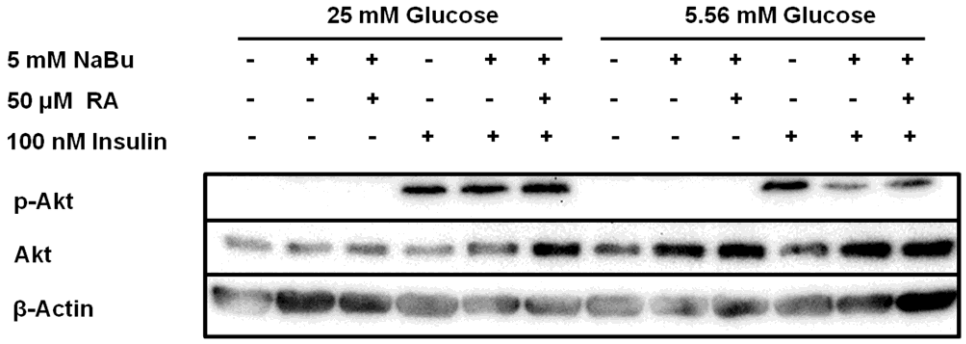


**Figure 7.** Insulin signalling pathway activation in HT-29 cells, grown in culture medium containing different glucose concentrations in the presence or absence of 100 nM insulin (30 min of incubation). Representative blots and corresponding loading control (β-Actin) are also presented.

Insulin did not alter the intracellular location nor the protein expression levels of both 80 kDa and 75 kDa SGLT1 bands in both glucose medium (data not shown). Furthermore, GLUT2 protein expression and intracellular location did not seem to be affected by insulin treatment (data not shown).

*3.5. Glucose and insulin did not affect SGLT1 expression in differentiated Caco-2 cells*

To investigate whether Caco-2 cells resemble a good *in vitro* model for studying the intestinal SGLT1 response to glucose and/or insulin, spontaneously differentiated Caco-2 cells were pre-incubated in a glucose-free solution (Krebs solution) for 30 min. Cells were then incubated for 30 min in Krebs solution (glucose-free or containing 25 mM glucose) in presence or absence of 100 nM insulin. SGLT1 and GLUT2 proteins display a similar intracellular location to the one found in HT-29 cells. However, neither glucose nor insulin affected significantly the levels of protein and their intracellular location in Caco-2 cells (**Figure 8**). Despite the short time used in this assay, we expected to observe some changes at least on the intracellular location of SGLT1, since little time is needed to modify protein trafficking.

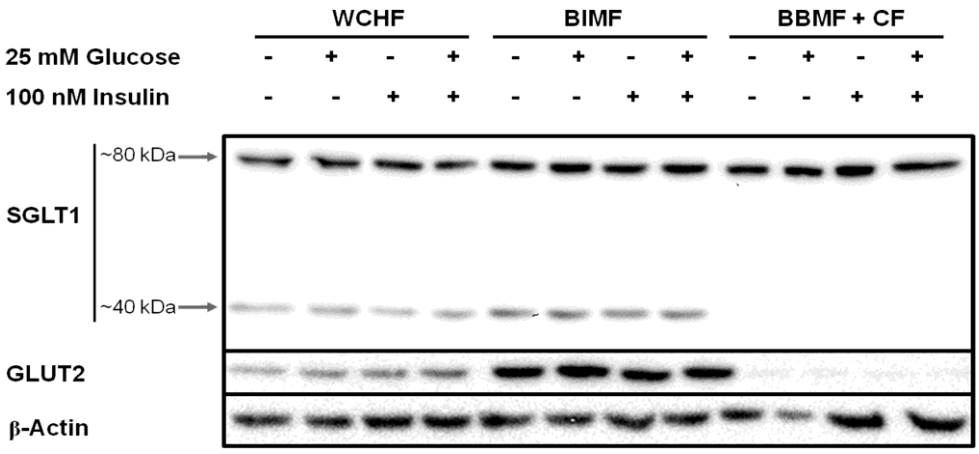


**Figure 8.** Effects of high levels (25 mM) of glucose and 100 nM insulin on SGLT1 and GLUT2 expression in subcellular fractions of differentiated Caco-2 cells. Differentiated Caco-2 cells were pre-incubated for 30 min in glucose-free Krebs solution followed by incubation with refreshed glucose-free or 25 mM glucose Krebs solution in the presence or absence of 100 nM insulin, for other 30 min. β-Actin was used as loading control.

## Discussion

Enterocyte's maturation and differentiation is a complex process that begins with the proliferation and differentiation of stem cells in the crypts. These progenitors will then migrate and maturate towards the villus tip and extrusion of senescent cells will occur (Carethers, 1998; Ferraris, 2001).

In recent years, human colonic Caco-2 and HT-29 cells have been widely used in many areas of pharmacology and toxicology research as an alternative to resource and time-consuming *in vivo* studies (Artursson et al., 2001). Once differentiated, both Caco-2 and HT-29 cells exhibit high levels of enzymes characteristic of the enterocyte's BBM such ALP. Whereas Caco-2 differentiate spontaneously in culture, forming polarized cell monolayers connected by tight junctions and expressing structural and functional characteristics of mature enterocytes (Pinto et al., 1983), HT-29 cells grow in standard conditions as unpolarized and undifferentiated cells, lacking the expression of enterocytic markers. However, this cell line is able to undergo differentiation following some modifications of culture medium (glucose deprivation and use of galactose as carbon source) or using differentiation agents (butyrate) (Simon-Assmann et al., 2007). Butyrate is a short-chain fatty acid produced in the colonic lumen by bacterial fermentation of dietary fibres and carbohydrates. Many reports have shown that butyrate inhibits cell proliferation and regulates cell cycle progression through inducing p21

expression that leads to cyclins and cdks inhibition and culminates with a blockage in G1 phase (Siavoshian et al., 1997). Downregulation of c-*myc* expression is also a feature of butyrate (Barnard and Warwick, 1993). With the present study, we aimed to characterize the response of differentiated Caco-2 and HT-29 cells to the concentration of glucose in the medium in terms of intracellular location and protein expression of glucose transporters, namely SGLT1 protein, in order to choose the best *in vitro* model for intestinal dietary adaptation studies. A low glucose concentration in the medium did not affect cell growth of Caco-2 cells but did for HT-29 cells. After 48h with low glucose medium, cell growth decreased dramatically, probably due to induction of differentiation by a low glucose concentration. NaBu treatment in HT-29 cells grown in low glucose did not increase so much the differentiation marker ALP, which agrees with the fact that cells growing in low glucose are probably already with some degree of differentiation. In high glucose concentration, as expected, NaBu increased ALP activity as well as p21 and p27 protein levels in HT-29 cells, confirming the ability of butyrate to induce cell cycle blockage and differentiation in this cell line. As reported by Pinto and colleagues (1983), Caco-2 cells achieved full differentiation within 20 days, as indicated by ALP activity, when grown in high glucose medium. However, when cells were growing in 5.56 mM glucose medium, no increase of ALP activity was observed after 14 days, which may indicate that differentiation in these conditions is faster, although the activity of ALP is lower when compared with that grown in high glucose levels.

Enterocytes express proteins such as SGLT1 in matching amounts to the glucose freed by digestion of carbohydrates. Therefore, diets rich in digestible carbohydrates induce high SGLT1 protein expression on BBM of enterocyte than diets low in digestible carbohydrates (Diamond et al., 1984). In addition to the response to diet, disease processes such as diabetes mellitus are also characterized by higher than normal BBM SGLT1 expression (Burant et al., 1994; Dyer et al., 2002). Interestingly, the increase of SGLT1 levels after differentiation with NaBu was significantly higher in cells cultured in high glucose medium, which may reflect the well known effects of increasing amounts of digestible carbohydrates and also diabetes on this glucose cotransporter (Chang et al., 2007; Engle et al., 1998; Hodin et al., 1996; Wang et al., 2001). These evidences suggest that HT-29 seem to respond to differentiation events that resembles enterocytes and to promote the *in vivo* glucose-induced intracellular SGLT1 redistribution. However, our SGLT1 data raised some doubts about the

maturation status of the protein in HT-29 cells since a peculiar pattern was observed, in which two bands with different molecular weight (80 kDa and 75 kDa) and intensity appeared even using different SGLT1 antibodies. It is known that maturated SGLT1 contains 14 transmembrane α-helices with extracellular N and C terminus, a unique glycosilation site and two phosphorylation sites (Wright, 1998). Addressing the possibility that this pattern reflects a glycosilation state of SGLT1 in HT-29 cells, we performed a deglycosilation assay that revealed that neither HT-29 nor Caco-2 seem to have glycosilated SGLT1. Hence, this two-band pattern may reflect other post-translational modifications or correspond to the matured SGLT1 protein of colonocytes. Only the 80 kDa SGLT1 band (upper band) was significantly modified by NaBu-treatment, suggesting that this band is probably the one that mostly resemble the enterocytic response to dietary factors. NaBu-treatment modified the intracellular location of SGLT1 protein; it removed the glucose cotransporter from the BIMF (where it is probably associated with intracellular vesicles) and promoted the insertion of SGLT1 into the BBM fraction. GLUT2 protein expression in whole HT-29 cells lysates was induced by butyrate independently on glucose concentration. Additionally, our subcellular procedure revealed that GLUT2 was located preferably on the BIMF which contains basolateral membranes, even under prolonged exposure to high glucose conditions. These evidences seem to contradict Kellet and Laroche (2005) studies that showed an apical location of GLUT2 in enterocytes of several experimental models under increased luminal concentration of glucose or fructose. They also found that little GLUT2 is expressed in apical membranes under low luminal glucose (Kellett and Brot-Laroche, 2005). As expected, RA did not alter the total protein expression levels of SGLT1 and GLUT2, however, no inhibition of the glucose-induced increase of SGLT1 levels at BBMF in NaBu-differentiated HT-29 cells was observed, contrasting with our previous *in vivo* study (Azevedo et al., 2011). This may indicate that the ability of RA to decrease SGLT1 levels in BBM *in vivo* may not involve directly the modulation of SGLT1 trafficking but probably of the digestive enzymes that freely glucose from amido of the diet since RA was found to be a competitive inhibitor of yeast α-glucosidase (Kwon et al., 2006; Lin et al., 2011) and to inhibit porcine pancreatic amylase *in vitro* (McCue and Shetty, 2004). Other hypothesis is that NaBu-induced differentiation in HT-29 cells does not resemble the physiologic and cellular mechanisms of enterocytes *in vivo*, and therefore is inappropriate to study the regulation of SGLT1 by dietary constituents.

Despite the pivotal role of insulin on peripheral glucose uptake, little is known about the involvement of insulin on the regulation of intestinal SGLT1 activity and expression. It has been reported that subcutaneous insulin treatment in diabetic rats, with increased BBM SGLT1 expression, significantly decreased SGLT1 levels without changes in mRNA levels, suggesting a mechanism involving vesicle trafficking (Kurokawa et al., 1995). A more recent experiment, performed both in rats and Caco-2 cells, showed that insulin stimulates intestinal glucose transport through increasing the number of SGLT1 transporters and decreasing the activity and protein levels of $Na^+/K^+$-ATPase (Serhan and Kreydiyyeh, 2010). Our results showed that in Caco-2 cells, SGLT1 and GLUT2 transporters had a similar intracellular location to the one found in HT-29 cells but neither glucose nor insulin seems to significantly alter the total expression and cellular distribution of both transporters, after 30 min exposure. Moreover, insulin did not alter the intracellular location nor protein expression levels of SGLT1 and GLUT2 in HT-29 cells. Since that, in polarized cells, sorting of proteins occurs in the trans-Golgi network and they are rapidly internalized in appropriate cellular compartment, short time incubations with possible regulators of this network should be effective. In this experiment, that was not the case. Our evidences are in concordance with the previous data reported that extracellular D-glucose exposure did not alter the cellular redistribution of SGLT1 in Caco-2 cells (Khoursandi et al., 2004).

Our results showed that SGLT1 and GLUT2 transporters of NaBu-differentiated HT-29 cells respond to glucose stimulation, resembling the *in vivo* situation. However, we were not able to mimic *in vitro* effect of RA on SGLT1 expression that we find *in vivo*, which may indicate that it did not depend directly on the modulation by glucose levels in the luminal space. Despite the undeniable value of Caco-2 and HT-29 cells in delineating potential pathways related to enterocyte differentiation and predicting intestinal absorption of drugs, further work needs to be performed using alternative experimental models and/or approaches to clarify the previous observed effects of RA on BBM SGLT1 expression and blood glucose.

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# CHAPTER 6

## *Final Considerations*

## 6.1. General discussion and conclusions

The most pertinent results presented in detail in the course of this thesis will be briefly discussed in this section.

Currently, there is a renewal interest in the use of natural compounds to treat and control diabetes and dyslipidemia. These natural products have been used since antiquity and are considered economical and safe alternatives than the currently available drugs. The work herein described meant to emphasise the value of *Salvia fruticosa* and *Salvia officinalis* water extracts (prepared as tea) and some of their isolated compounds as potential antidiabetic and lipid-lowering agents.

*Chapter 2* describes a pilot trial performed to elucidate the effects of *S. officinalis* tea (SOT) on glucose regulation in healthy female volunteers that belong to an age group at risk for developing T2DM. The results demonstrated that sage tea drinking did not induce hepatotoxicity or other side effects (such as on blood pressure and on body weight). In addition, hypoglycaemias may be also excluded since no effects on fasting and/or postpandrial blood glucose levels were observed. Sage treatment strengthened the erythrocytes' antioxidant status through increasing SOD and CAT activities, which may indicate its potential to prevent against cellular oxidative damages and LDL cholesterol oxidation. This potential in preventing CVDs progression was reflected in the amelioration of the plasma lipid profile of the volunteers, where an increase in HDL levels (by 50.6% at the end of the treatment and 37.6% after two weeks wash-out) was observed. In addition, a gradual decrease on total and LDL cholesterol levels was also detected. Recently, a clinical trial corroborated our findings and added supplementary evidences, by demonstrating that *S. officinalis* tea decreased triglycerides and VLDL levels without inducing hepatic and renal toxicity (Kianbakht et al., 2011).

*Salvia fruticosa* tea (SFT) also showed to increase plasma HDL cholesterol levels (by 21%) after the reintroduction of a normal (referred as high carbohydrate, [Hc]) diet (c*hapter 3*). It has been reported that nicotinic acid (niacin), the most powerful agent presently available for correcting HDL cholesterol levels, induces a 15%-35% increase in HDL levels (Toth et al., 2005). In addition, other lipid-lowering agents such as fibrates and statins, caused only a 10%-15% and 5%-15% increase, respectively (Toth et al., 2005). In spite of this beneficial effect, several adverse effects and contraindications have been reported for these drugs (Mooradian, 2009). Thus, the

effects of SFT and particularly SOT on plasma lipid profile reveals promising in the search for more reliable and safe alternatives to these pharmacological therapies.

Evidences from a previous *in vivo* work demonstrated that treating rats with SFT, during the four days of Hc diet reintroduction, significantly reduced the adaptive increase of SGLT1 expression in BBM (Azevedo et al., 2011). Rosmarinic acid (RA), the major phenolic compound of both sage extracts (representing 70% of all phenolic compounds present in SOT and 72% in SFT, see **Table 4**, *chapter 1*), was proposed to be the active principle behind those beneficial properties of SFT (Azevedo et al., 2011). However, the beneficial effects of both sage extracts on plasma lipoproteins did not seem to be due to RA, since its aqueous extract did not modify plasma lipid profile (*chapter 3*). This may be related with the observed RA-mediated downregulation of LDLR expression, the receptor responsible for cholesterol clearance from circulation, which was not affected by SFT drinking. Nevertheless, RA seems to potentially 1) regulate dietary cholesterol absorption, by preventing the carbohydrate-induced increase of BBM NPC1L1 transporter, a similar outcome to ezetimibe; 2) control hepatic cholesterol *de novo* synthesis, through downregulation of HMGCR mRNA levels; 3) promote hepatic fatty acid catabolism, through increasing the expression of PPARα, and its target gene CPT1 and 4) prevent fatty acid synthesis, through decreasing SREBP-1 gene expression and FAS protein levels. SFT also showed a beneficial prevention of fatty acid synthesis by decreasing FAS expression in the liver as a probable consequence of the observed SFT-mediated reduction of ChREBP mRNA levels. The overall favourable properties of both SFT and RA drinking make them auspicious agents for maintaining lipid homoeostasis and prevent disease onset. However, the data here presented disclose that distinct mechanisms of action and pathways are modulated by these natural products. In addition, RA does not seem to be the active principle responsible for the beneficial effects of both sage plants in plasma lipoproteins.

In addition to RA, the flavonoids luteolin-7-glucoside (L7G) and 6-hydroxyluteolin-7-glucoside, are also present in sage extracts (representing 26% of all phenolic compounds present in SOT and 13% in SFT, see **Table 4**, *chapter 1*). A previous study performed in healthy rats demonstrated that dietary supplementation with L7G lowered plasma glucose (from 9.78 mM to 8.78 mM) and decreased plasma total and LDL cholesterol levels (by 29.2% and 39.5%, respectively) (Azevedo et al., 2010). Intending to clarify the mechanism responsible for this improvement on lipid profile, L7G was assessed *in vivo* in healthy rats regarding effects on lipid metabolism

(*chapter 4*). L7G showed to significantly increase both PPARα and CPT1 gene expression in the liver (by 1.9 and 1.8 fold, respectively), in a similar but less prominent way than RA (which promoted a 3.1 and 5.1 fold increase, respectively). Moreover, L7G-supplemented diet was also effective in decreasing hepatic HMGCR gene expression without affecting SREBP-2 and LDLR mRNA levels, a similar outcome to SFT. These data seem to indicate that additional pathways may modulate LDLR and HMGCR transcriptional activation. The potential effect of L7G for blocking SREBP-1 maturation and/or activity and activating ER stress-related pathways, by upregulating GRP78/BIP expression, require further investigation. Although, these data may suggest an effect of L7G in activating transient ER stress pathways aimed at restoring cellular homeostasis. Overall, the results seem to indicate an involvement of this phytochemical on the reported cholesterol-lowering effects of SOT (*chapter 2*). Moreover, the absence of L7G in *S. fruticosa* extract, may explain the lack of effects of SFT on total and LDL cholesterol levels (*chapter 3*). The presence of other compounds, even at lower concentrations, or the mixture of them in both sage extracts may contribute to the observed improvement on plasma lipid profile.

Finally, aimed at establishing an *in vitro* model to study the molecular mechanisms behind the intestinal SGLT1 regulation in response to dietary constituents, such as glucose and phytochemicals, the experiment described in *chapter 5* was performed. Among all the current available intestinal cell lines, butyrate (NaBu)-induced differentiated HT-29 cells and spontaneously differentiated Caco-2 cells were selected due to their well recognized enterocyte-like characteristics. Glucose concentration in culture medium seemed to affect differently the cellular growth and differentiation status of both cell lines. While glucose levels in culture medium did not seem to affect Caco-2 cells growth, low glucose levels seemed to favour the cellular growth (within 48 h) and cellular differentiation (within 72 h) of HT-29 cells. Under high glucose levels, NaBu induced an increase in SGLT1 and GLUT2 protein expression levels in HT-29 whole cell homogenates. Moreover, this short-chain fatty acid promoted an enhancement on BBM SGLT1 levels and a simultaneous increase of GLUT2 expression in the remaining membrane fraction of HT-29 cells. These evidences suggest that HT-29 cells responds to differentiation events that resembles enterocytes and endorses the well reported effects of digestible carbohydrates on the intracellular redistribution of intestinal SGLT1 (Azevedo et al., 2011; Ferraris 2001). NaBu-differentiated HT-29 cells were treated with RA, in an attempt to confirm the

reported *in vivo* effects of this phytochemical on BBM SGLT1 expression (Azevedo et al., 2011). However, RA did not prevent the glucose-stimulated enhancement of SGLT1 at the BBM of HT-29 cells. Furthermore, the protein levels and location of SGLT1 in these cells did not seem to be affected by insulin. A similar localization of SGLT1 and GLUT2 transporters was observed in spontaneously differentiated Caco-2 cells, but the protein levels of these transporters were not changed by either glucose or/and insulin exposure. Thus, the potential role of insulin on the regulation of intestinal SGLT1 still requires clarification. Taken together, these evidences seem to point out some limitations of employing HT-29 and Caco-2 cells as *in vitro* models for studying the regulation of SGLT1 by dietary constituents, namely RA, although both cell lines are recognized tools for drug absorption studies.

In summary, this thesis is an important contribution to elucidate the biological properties of both sage species and some of their isolated natural compounds, in mechanisms related to glucose and lipid metabolism. Medicinal herbs are empirically used since ancient times to treated multiple disorders still, the easily access with limited information regarding potential side effects, makes them unattractive therapeutic tools for clinical practice. To assist the implementation of these sage products as safe and reliable therapeutical drugs for preventing diabetes and other related diseases, additional studies must be conducted.

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## 6.2. Future perspectives

The studies presented in this dissertation were conducted to characterize potential antidiabetic and lipid-lowering effects of selected sage species (*Salvia fruticosa* and *Salvia officinalis*), and to describe the molecular mechanisms of action that support those beneficial properties. The identification of new active compounds with therapeutic properties was an additional goal. Notwithstanding the contribution of this work to the elucidation of those topics, additional and complementary research must be conducted to clarify the unanswered questions.

The efficacy of *S. officinalis* extract to improve antioxidant defences and lipid profile of healthy humans without causing toxicity, paves the way for testing sage's effects in a population with predisposition to develop diabetes. These new experiments must also take into account the need of interrupted sage tea treatments, with short or long periods of time, to improve health condition. Interesting would be to carry out this experiment in diabetic patients, where the true antidiabetic potential of sage would be tested. However, multiple cautions would need to be considered to avoid unexpected side effects that aggravate their diabetic condition.

Since CVDs constitute the leading cause of death worldwide and are the major cause of morbidity and mortality in diabetic patients, efforts must be made in order to strive against this reality. The molecular mechanisms behind the beneficial effect of *S. fruticosa* and particularly *S. officinalis* extracts on HLD cholesterol levels must be investigated concerning several potential targets, such as enzymes of the reverse cholesterol transport pathway. The potential for sage plants and their compounds modulates the expression of nuclear receptors and bile acid-regulated genes, involved in bile acid synthesis, transport and cholesterol metabolism, must also be explored. Since diet constitute an important risk factor in disease progression, it will be interesting to evaluate the effects of these phytochemicals in animals fed a high-fat diet, to better characterize their lipid-lowering potential.

It will be also interesting to compare the effects of L7G and its aglycone luteolin in cultured hepatocytes (primary cultures and/or HepG2 cells), in several parameters relevant to lipid metabolism. Comparing the effects of those natural compounds with the available pharmacological lipid-lowering drugs, such as statins, ezetimibe and

fibrates should also be considered, in order to find more reliable and safe therapies than the ones currently used.

As a final point and since the majority of human cell lines are derived from tumours and miss the physiological environment found *in vivo*, it was not surprising to find that differentiated HT-29 and Caco-2 cells offered some limitations for studying SGLT1 regulation in response to dietary constituents. Thus, new *in vitro* strategies (such as everted gut sac or primary cultures of enterocytes) must be considered in order to obtain more reliable data to the *in vivo* situation. The lack of relevant data concerning to the effects of insulin on the regulation of the intestinal SGLT1 expression should be explored by using both *in vivo* and *in vitro* models. However, it would not be surprising to find distinct insulin outcomes in the distinct models. Given the importance of intestinal SGLT1 on glucose absorption and the role of the small intestine in glucose and cholesterol homeostasis, this organ is an important target for antidiabetic and lipid-lowering therapeutical interventions.

In conclusion, the results presented in this thesis and the elucidation of the unanswered questions, will certainly contribute to the identification of new compounds that serves as therapeutic tools for diabetes and its associated complications.