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Diet and exercise – impact on lipid metabolism and involvement of epigenetic mechanisms

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Work performed under the orientation of:
Prof. Dr. Cristina Pereira Wilson
Prof. Dr. Maria João Martins

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DECLARAÇÃO

Nome: Ana Rita Pereira Oliveira
Endereço eletrónico: ana.rpo@hotmail.com
Telefone: 912028476
Número do Cartão de Cidadão: 13986221 8 ZZ5

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Orientadores: Professora Doutora Cristina Pereira-Wilson;
Professora Doutora Maria João Martins.

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Diet and exercise – impact on lipid metabolism and envolvement of epigenetic regulation

Abstract

A sedentary lifestyle and consumption of high-caloric and micronutrient pour diets are strong risk factors associated with the development of cardiovascular diseases, obesity, dyslipidemia and colon cancer, where deregulation of cholesterol metabolism plays an important role. This thesis is divided in two experimental parts: Part A: *Effects of exercise on cholesterol metabolism in high-fat fed rats: involvement of epigenetic mechanisms*; and Part B: *Effects of naturally occurring dietary flavones on liver cholesterol metabolism, in vitro.*

In our study (Part A), were analysed colon samples from *Sprague-Dawley* rats submitted to 2 iso-hipercaloric diets: standard diet (35 % of calories derived from fat), and high-fat diet (71 % of calories derived from fat), and each group further divided in 3 sub-groups according to the exercise regime: sedentary, voluntary physical activity and endurance training. The consumption of a high fat diet significantly increased global methylation levels. However, in both diets DNA methylation seem to be normalized by voluntary exercise, while endurance training had no effect. Our qRT-PCR analysis did not show differences in *SREBP-2* and *ABCA1* expression. However, high-fat consumption increased gene expression of the efflux transporter of neutral sterols, *ABCG5*, which was reverted by voluntary physical activity. Our study diets seem not have an effect on *NPC1L1* expression, but voluntary exercise in both diets, and endurance training in standard diet, decreases *NPC1L1* expression, decreasing cholesterol re-absorption capacity.

In our study (Part B), we assessed the effect of Luteolin (L) and its major naturally occurring glycosylated form, Luteolin-7-O-Glucoside (L7G), on cholesterol metabolism regulation *in vitro* using human hepatocellular carcinoma HepG2 cells. Our work show that L reduces both SREBP-2 activation and *HMGCR* gene expression, similarly to simvastatin, revealing an effect on cholesterol endogenous synthesis regulation. In addition, both L and L7G seem to increase PPARα protein levels. The results suggest the potential effect of flavones, L and L7G, in the prevention of abnormal cholesterol metabolism. The combination of these flavones consumption, with regular physical activity and reduced fat intake may prevent metabolic related diseases.

Dieta e exercício – impacto no metabolismo lipídico e envolvimento de regulação epigenética

Resumo

Um estilo de vida sedentário e o consumo de uma dieta hipercalórica e pobre em micronutrientes são fortes fatores de risco para o desenvolvimento de doenças cardiovasculares, obesidade, dislipedimea e cancro colorectal, nas quais a desregulação do metabolismo do colesterol tem um papel importante. Esta tese está dividida em duas partes experimentais: Parte A: *Efeitos do exercício físico no metabolismo do colesterol em ratos alimentados com altos teores de gordura: envolvimento de mecanismos epigenéticos*; e Parte B: *Efeitos de flavonas naturais presentes na dieta, no metabolismo do colesterol, in vitro.*.

No nosso estudo (Parte A), foram analisadas amostras de colon de ratos *Sprague-Dawley* submetidos a 2 dietas iso-hipercalóricas: dieta padrão (35 % das calorias derivadas de gordura), e dieta rica em gorduras (71 % das calorias derivadas de gordura), cada grupo foi ainda dividido em 3 sub-grupos de acordo com o regime de exercício: sedentários, atividade física voluntária e treino de resistência. O consumo de uma dieta rica em gorduras aumentou o nível de metilação global. No entanto, nas duas dietas, a metilação do DNA parece ser normalizada pela prática voluntária de exercício físico. Na nossa análise, por qRT-PCR, não foram encontradas diferenças na expressão de *SREBP-2* e *ABCA1*. Contudo, o consume de muita gordura aumentou a expressão do transportador de efluxo de esteróis neutros, *ABCG5*, efeito que foi contrariado pela prática de atividade física voluntária. As dietas deste estudo não parecem afectar a expressão de *NPC1L1*, mas o exercício voluntário nas duas dietas, e treino de resistência na dieta padrão reduziram a expressão de *NPC1L1*, diminuindo a capacidade de re-absorção de cholesterol.

No nosso estudo (Parte B), foram analisados os efeitos da Luteolina (L) e a sua forma glicosilada com maior ocorrência natural, Luteolina-7-O-Glicosideo, no metabolismo do colesterol *in vitro*, usando células HepG2 de carcinoma hepatocelular humano. O nosso trabalho mostrou que L reduz tanto a clivagem do SREBP-2, mas também a expressão de *HMGCR*, de forma similar à sinvastatina, revelando um efeito na regulação da síntese endógena do colesterol. Ainda, tanto a L como L7G parecem aumentar os níveis de proteína do PPARα. Este trabalho mostrou o potencial de flavonas, L e L7G, na prevenção de doenças relacionadas com o metabolismo do colesterol. A combinação do consumo de flavonas, com a prática regular de exercício e redução da ingestão de gordura podem prevenir doenças metabólicas.

Contents

List of abbreviations

ABC – ATP Bindig Cassete – ABC;

ACAT – Acyl-CoA: Cholesterol Acyltransferase;

ACC – Acetyl-CoA Carboxylase;

AHA – American Heart Association;

AMPK – 5-Adenosine Monophosphate-Activated Protein Kinase;

BSA – Bovine Serum Albumin;

CHD – Coronary Heart Disease;

CPT1 – Carnitine Palmitoyltransferase 1;

CVD – Cardiovascular Diseases;

CYP7A1 – Cytochrome P450 Enzyme Cholesterol 7 α-hydrolase;

DMSO – Dimethyl Sulfoxide;

DNMT – DNA Methyltransferases;

EDTA – Ethylenediaminetetraacetic Acid;

FA – Fatty Acids;

FAS – Fatty Acid Synthase;

FBS – Fetal Bovine Serum;

FF – Fenofibrate;

FXR – Farnesoid X Receptor;

HAT – Histone Acetyltransferases;

HDAC – Histone Deacetylases;

HDL – High Density Lipoproteins;

HMGCR – 3-Hydroxy-3-Methylglutaryl-Co Enzyme A Redutase;

HMGCS – 3-Hydroxy-3-Methylglutaryl-Co Enzyme A Synthase;

HS – High-fat diet, sedentary;

HT – High-fat diet, endurance training;

HV – High-fat diet, Voluntary exercise;

IDF – International Diabetes Federation;

INSIG – Insulin Induced Gene;

L – Luteolin;

L7G – Luteolin-7-O-Glucoside;

LXR – Liver X receptor;

LXRE – LXR responsive elements;

MEM – Minimum Essential Medium Eagle;

MetS – Metabolic Syndrome;

MOPS – 3-(N-Morpholino)propanesulfonic acid;

MTT – 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide;

MTTP – Microsomal Triglyceride Transfer Protein;

NAFLD – Non-alcoholic Fatty Liver Disease;

NASH – Non-alcoholic Steatohepatitis;

NCBI – National Centre for Biotechnology Information;

NHLBI – National Heart, Lung, and Blood Institute;

NPC1 – Niemann-Pick C1;

NPC1L1 – Niemann-Pick C1-Like 1 – NPC1L1;

PBS – Phosphate-buffered saline;

PEPCK – Phosphoenolpyruvate Carboxykinase;

PPAR – Peroxisome Proliferator-Activated Receptor;

qRT-PCR – quantitative Real Time – Polymerase Chain Reaction;

RXR – Retinoid X Receptor;

SAH – S-Adenosylhomocysteine;

SAM – S-Adenosylmethionine;

SCAP – SREBP Cleavage Activating Protein;

SIMV – Simvastatin;

SR-BI – Scavenger Receptor class B type I;

SREBP – Sterol Regulatory Element Binding Protein;

SS – Standard diet, sedentary;

SSD – Sterol Sensing Domain;

ST – Standard diet, endurance training;

SV – Standard diet, voluntary exercise;

T2DM – Type II Diabetes Mellitus;

TCC – Total Cellular Cholesterol;

VLDL – Very Low-Density Lipoproteins.

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Chapter 3

Objectives and scope of the thesis

This master thesis focus on aspects of lifestyle, such as diet and physical activity, on health. Diet and physical activity are important features that we can control and adapt according to our needs and preferences. Additionally, they are an useful tool in the prevention and treatment of pathologies, namely metabolic diseases. Cardiovascular diseases, type II diabetes mellitus and non-alcoholic fatty liver disease, are pathologies very influenced by lifestyle factors, and collectively referred to as components and associated features or consequences of the Metabolic Syndrome. The incidence numbers are rising, and Metabolic Syndrome related diseases are currently a huge public health challenge.

This work is divided in two distinct experimental parts.

In Chapter 2 – Experimental Part A: *Effects of exercise on cholesterol metabolism in high-fat fed rats: involvement of epigenetic mechanisms*, we aim to understand the effect of a high-fat diet combined with different types of exercise (voluntary and endurance) on intestinal cholesterol transport and metabolism. Briefly, using colon samples from *Sprague-Dawley* rats submitted to different diets and exercise regimens, we analysed the expression of transporters related to cholesterol absorption and secretion, and the main regulators of cholesterol synthesis. Epigenetic marks are affected by lifestyle and environmental factors, and may be a key factor to define susceptibility to diseases. Therefore, we also intended to evaluate epigenetics changes, namely total DNA methylation.

In Chapter 3 – Experimental Part B: *Effects of naturally occurring dietary flavones on liver cholesterol metabolism, in vitro*, we proposed to evaluate the effect of natural compounds on lipid metabolism, using an *in vitro* approach. We assessed the effect of Luteolin and its major natural occurring form, Luteolin-7-O-Glucoside, *in vitro* using human hepatocellular carcinoma HepG2 cell line. Luteolin is a flavonoid and a member of the flavone sub-family and has been associated to a great number of biological activities, which includes lipid metabolism modulation. We intended to evaluate these compounds since they are abundant in fruits and vegetables, which consumption is easily added (or increased) in the human diet. Achieving a regulation of lipid metabolism would be essential as a preventive measure against metabolic diseases, and specifically non-alcoholic fatty liver disease.

Chapter 1 - General Introduction

1.1 - Lifestyle Related Diseases

Our health is highly influenced by our way of living: what we eat, how we behave and how active we are, combined with individual genetic characteristics, determine our susceptibility to diseases. Metabolic diseases are an example of pathologies in which prevention includes a balanced diet and an improvement of physical activity (Kirchner et al., 2012; WHO/FAO, 2003).

However, a sedentary lifestyle and a non-varied and hypercaloric diet are typical of the Western world. The economic development with industrialization and market globalization improved the standards of living by increasing access to services and food availability. But these changes led to an increase of food consumption, side by side with an increase of diets high in fat and low in unrefined carbohydrates, vegetables and fruits. Also led to modifications in the kind of work, which, currently, is less physically demanding contributing to a sedentary pattern. In combination, all these changes have contributed to the increase of chronic diseases such as obesity, type II diabetes mellitus (T2DM), cardiovascular diseases (CVD), hypertension, stroke and some types of cancer, as colorectal cancer (WHO/FAO, 2003). In particular CVD has been classified as the most important cause of morbidity and mortality worldwide, being a major public health challenge. It is important to notice that the majority of the risk factors for the development of CVD, such as high body weight, hypertension, insulin resistance and dyslipidaemia, constitute the Metabolic Syndrome (MetS) and are also related to the chronic diseases mentioned (Afzali et al., 2013).

1.1.1 - The Metabolic Syndrome

The features grouped as MetS share interconnected physiological, biochemical, clinical, and metabolic factors, which include, as mentioned above, visceral adiposity accumulation, hypertension, dyslipidaemia and glucose intolerance, leading to an increased risk for development of CVD and T2DM. Since the first time that MetS was defined, in 1920 by Kylin, many criteria have been used to define this term, being necessary to achieve a consensus (Kaur, 2014). In *Table 1.1* are described the criteria brought together by an initiative of major health organizations, including International Diabetes Federation (IDF), National Heart, Lung, and Blood Institute (NHLBI) and American Heart Association (AHA), to be considered in the diagnosis of MetS (Alberti et al., 2009). The occurrence of three out of the five criteria results in MetS. Currently the cut point to waist

circumference as a measure for central obesity is ethnic/racial specific, since the norm for body weight distribution varies with ethnicities and nationalities (IDF, 2006; Kaur, 2014). Pharmacological treatment to control raised triglycerides and reduced high-density lipoproteins (HDL) cholesterol, or previously diagnosed hypertension or T2DM weights the same in definition as the abnormal values (Alberti et al., 2009; Eckel et al., 2010). According to the IDF definition (2006) it was estimated that 25 % of the world's adult population has MetS. However in some regions of the globe the number can be 84 % (Cornier et al., 2008; Kaur, 2014).

Table 1.1 – Criteria that define the Metabolic Syndrome. According to (Alberti et al., 2009).

Criteria	***Cut points***
Central obesity	Waist circumference ≥ 94 cm for Europid men; ≥ 80 cm for Europid women
Triglycerides	≥ 150 mg/dL (1.7 mmol/L)
HDL cholesterol	Men < 40 mg/dL (1.03 mmol/L); Women < 50 mg/dL (1.29 mmol/L)
Blood pressure	Systolic ≥ 130 mm Hg or Diastolic ≥ 85 mm Hg
Fasting plasma glucose	≥ 100 mg/dL (5.6 mmol/L)

Several studies have been assessing the correlation between MetS and prediction of CVD and T2DM. And a higher number of abnormal factors in the MetS definition was strongly related with diabetes incidence (Ford, 2005; Ford et al., 2008). A systematic review and meta-analysis conducted by Mottillo and collaborators, in 2010, using 87 published prospective observational studies identified an association of MetS diagnosis with a 2-fold increase risk for CVD and severe complications, myocardial infarction and stroke, and also a 1.5-fold increase for all-cause mortality. The greater risk for CVD is not entirely dependent on the impairment of glucose metabolism in the definition of MetS (Mottillo et al., 2010). Despite an evident correlation between CVD and T2DM with MetS, there is a controversy about whether the diagnosis of MetS, as a cluster of risk factors, worsens the prognosis of metabolic related diseases relatively to the sum of risks of the individual factors (Ford et al., 2008; Mottillo et al., 2010).

1.1.2 - Non-alcoholic Fatty Liver Disease

Specifically in liver, MetS manifests as an increased input of free fatty acids (FA) into the liver, leading to fat accumulation and therefore fatty liver disease. FA could be originated from adipose tissue release, de novo biosynthesis and also from diet (Cohen et al., 2011). This condition is known as non-alcoholic fatty liver disease (NAFLD), and it is only diagnosed if there is no historic of heavy alcohol consumption: < 20 g/day in men and < 10 g/day in women, as well as absence of other liver diseases, such as Hepatitis B and C (Cauchy et al., 2014; Kim and Younossi, 2008).

Fatty liver, although not included, so far, in MetS definition, has been considered the first manifestation of MetS in the liver and the most common injury affecting the liver, being mainly associated with the increased obesity and T2DM development. Reaching about 30 % of the population in Western countries, NAFLD covers a range of conditions from simple steatosis to progressively worse diagnosis, such as non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (Cauchy et al., 2014; Cohen et al., 2011; Hassan et al., 2014; Kim and Younossi, 2008; Muhidin et al., 2012; Neuschwander-Tetri, 2005). The severity of NAFLD is distinguished by the liver parenchyma alterations in non-alcoholic fatty liver or simple steatosis - more than 5 % of hepatocytes containing fat - where there is no significant inflammation or liver fibrosis. And NASH, in which fat accumulation occurs in association with hepatic necroinflammatory changes, hepatocellular ballooning and fibrosis (Baran and Akyüz, 2014; Neuschwander-Tetri, 2005).

The progression to more severe states of NAFLD have been associated with risk factors encompassed in MetS, but fatty liver contributes itself to intensify the impairment of glucose metabolism, increased inflammatory response and insulin resistance. Despite association with other disorders directly related to liver, NAFLD has been also associated with colorectal cancer and CVD as an independent risk factor (Liu and Lu, 2014; Muhidin et al., 2012). This may be due to the drop in adiponectin level - an adipokine with anti-inflammatory properties -seen in NAFLD patients, which increases insulin levels worsening insulin resistance and promoting proliferative signals. The drop in the levels of adiponectin also contributes to the intensification of the pro-inflammatory state promoting angiogenesis what may contribute to tumour-cell proliferation in many cancers (Muhidin et al., 2012).

Insulin resistance leads to a greater lipolysis in adipocytes increasing plasma free FA. However, even in situations of insulin resistance, insulin does not seem to lose the capacity to induce lipogenesis through sterol regulatory element-binding proteins

(SREBP)-1c, as discussed later. Thus, the increased rate of FA synthesis leads to higher malonyl-CoA levels that inhibit FA β-oxidation leading greater fat accumulation in liver. Insulin resistance triggered by fat accumulation in the liver can be sufficient to induce dyslipidaemia, which is one of the criteria for MetS diagnosis. The liver exposure to free FA promotes chronic oxidative stress which may result in the degradation of apolipoprotein apoB100, necessary to very-low density lipoproteins (VLDL) assembly, and therefore reducing VLDL secretion and aggravating the hepatic steatosis (Gusdon et al., 2014; Koo, 2013).

Metabolic diseases have similar risk factors associated: physical inactivity, insulin resistance, hyperlipidemia, obesity, hypertension, and aging. Because of that, the diagnosis of a metabolic disease increases the likelihood of developing other related diseases since the pathological mechanisms are similar. In fact, aging is the only factor which we could not fight, although health-promoting behaviours, such as exercise and more diversified diet, lead to a healthier aging (WHO/FAO, 2003). As for MetS lifestyle related factors, nature of MetS has been associated to primary care for most of NAFLD patients, including dietary changes and exercise targeting significant weight loss. That is because the prevention of obesity also prevents MetS. Particularly, IDF recommends a moderate calorie restriction, an increase in physical activity and a change in dietary composition, aiming a decrease in 5 to 10 % of body weight (IDF, 2006; WHO/FAO, 2003). However, most patients may experience problems regarding long-term adherence to lifestyle interventions, and sometimes may regain the lost weight. Pharmacological alternatives are generally used to improve related conditions associated with the development of NAFLD (Baran and Akyüz, 2014).

1.2 - Lipid Metabolism and Regulation

As already mentioned metabolic diseases such as NAFLD and in particular CVD and T2DM have achieved record and epidemic proportions. The MetS cluster of factors is highly related to an abnormal lipid metabolism. On the other hand hepatic lipid and glucose metabolism are closely interrelated with lipid accumulation and FA β-oxidation is a central mechanism whose correct balance determines health maintenance. In addition, levels of cholesterol in plasma and mostly lipoprotein profile are crucial to prevent the development of such diseases (Bechmann et al., 2012). All these processes are regulated by hormones, such as insulin, nuclear receptors, such as peroxisome proliferator-activated

receptor (PPAR), liver X receptor (LXR), farnesoid X receptor (FXR) and transcription factors as SREBP (Eberlé et al., 2004; Fuchs, 2012; Gusdon et al., 2014; Ikonen, 2008).

1.2.1 - Cholesterol Synthesis and Absorption

As was already mentioned dyslipidaemia is strong risk factors to the development of metabolic diseases, thus cholesterol levels in association with plasma lipoproteins are elements that need to be tightly controlled. Dietary cholesterol is absorbed in the intestine and accounts to 60 % of total cholesterol. Since bile acids are produced only from cholesterol, biliary secretion of bile salts and their lost in faeces, when escaping the enterohepatic cycle, is relevant for the regulation of cholesterol synthesis (Barona and Fernandez, 2012; Hui et al., 2008; Meissner et al., 2010; Wang, 2003).

Nucleated cells have the capacity to synthesize cholesterol. Endogenous cholesterol synthesis begins with the condensation of three acetyl-coenzyme A units in the mevalonate pathway. The major limiting step of this pathway is catalysed by 3-hydroxy-3-methylglutaryl-coenzyme A redutase (HMGCR) which reduces HMG-CoA (3 acetil-CoA molecules condensed) leading to mevalonate formation, reaction in which two molecules of NADPH are consumed (Afzali et al., 2013; Ikonen, 2008; Li et al., 2010). *HMGCR* transcription is controlled mainly by SREBP-2. Studies using both animals and humans have showed that the levels of cholesterol that reach cells through low density lipoproteins (LDL)-mediated transport could reduce *HMGCR* expression, functioning as a compensatory pathway against excess dietary cholesterol (Barona and Fernandez, 2012). HMGCR enzyme is also inhibited by high concentrations of sterol and non-sterol metabolites products of the mevalonate pathway (Brown and Goldstein, 1980). Specifically, lanosterol, which is the first sterol intermediate in the cholesterol synthesis, seems to stimulate ubiquitination of HMGCR accelerating its degradation (Song et al., 2005).

HMGCR is also the target for statins, natural and synthetic pharmacological drugs, that inhibit the enzyme's activity and so blocking endogenous cholesterol synthesis (Wong and Dimitroulakos, 2002). Simvastatin (SIMV), one of the most used, seems to be capable to increase HDL and decrease LDL levels which could be related to a decrease in cardiovascular-related mortality. SIMV, additionally, seems to induce apolipoprotein apoA-I gene expression, contributing to HDL formation (Bonn et al., 2002). Statins are currently used as the most effective drugs in the struggle against CVD (Fu et al., 2014).

With regard to intestinal absorption, dietary cholesterol is absorbed from the lumen along the entire length of the small intestine, being the areas of duodenum and proximal jejunum the main sites (Wang, 2003). Its absorption occurs in a two-step process. First ,the insoluble cholesterol, as well as other lipids constituents of food are mixed with gastric enzymes and with bile and pancreatic juices, promoting the digestion and solubilisation of these constituents, including emulsion droplets formation and stabilization, which evolve to mixed micelles (Goodman, 2010; Hui et al., 2008; Wang, 2003). This step can be limited by the amount of bile acids synthetized and available to solubilisation, among other factors. The rate of bile acid production is limited by the transcriptional regulation of the cytochrome P450 enzyme cholesterol 7 α-hydrolase (CYP7A1), the rate limiting step of bile acid synthesis. An increase in bile acid levels in the liver leads to a down-regulation of their synthesis and stimulate their biliary secretion into the intestine, where they contribute decisively to lipid digestion and absorption. Bile acids/salts released may enter the enterohepatic circulation or be eliminated from the body in the stool, which is the highest form of the body sterol excretion (Li et al., 2013a; Wang, 2003). The presence of bile salts, lysophospholipids, phospholipids and monoacylglycerides, as well as free FA, increases cholesterol solubilisation/transport and, therefore, increases its absorption. In typical Western diet about 300 to 500 mg of cholesterol are consumed each day (Wang, 2003).

The second step of absorption occurs with the diffusion of mixed micelles through apical membrane of enterocytes by Niemann-Pick C1-like 1 (NPC1L1) transporter. Once in the endoplasmic reticulum, cholesterol is esterified through the action of acyl-CoA: cholesterol acyltransferase (ACAT) to be incorporated into nascent chylomicron (Hui et al., 2008; Wang, 2003, 2007). The transcription of ACAT seems to be dependent on the amount of cholesterol present, and is only active when sterols bind the activator allosteric site of the enzyme creating stimulatory structural modifications. The nascent chylomicrons are then loaded with neutral lipids by microsomal triglyceride transfer protein (MTTP) followed by incorporation of apolipoprotein B48, which is essential for chylomicrons maturation and secretion into the lymph (Li et al., 2010; Wang, 2003, 2007). A squamatisation of this process is presented in *Figure 1.1* that also shows the ABCG5/G8 dimer which limits sterol absorption, as discussed later.

The mature chylomicrons, the lipoprotein with lowest density, in circulation release FA into adipocytes, heart, skeletal muscle and lactating mammary tissue. Chylomicrons are endocytosed in the liver and the remaining lipids are repackaged into VLDL

which through endothelial lipoprotein lipase action releases FA. This produces a reduction in triacylglycerides levels in VLDL and originates LDL contributing to lipid distribution to peripheral tissues (Ikonen, 2008).

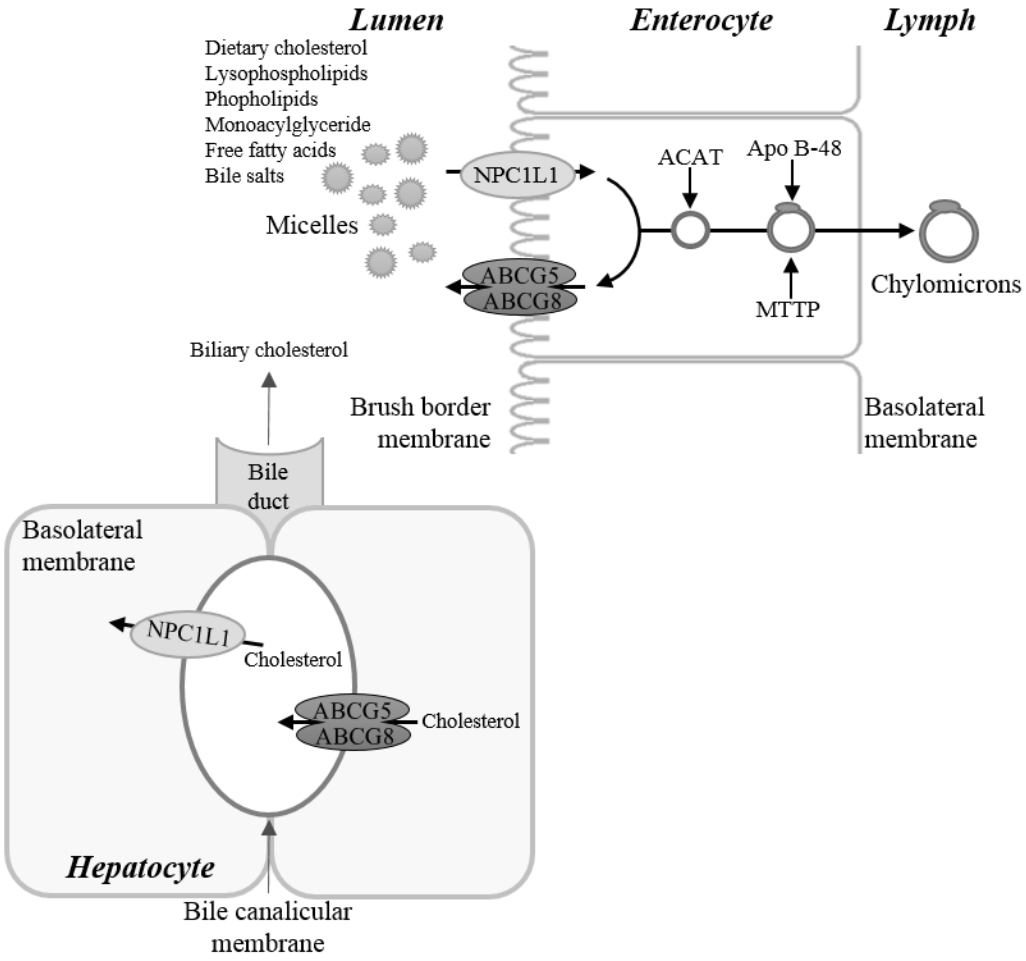


Figure 1.1 – Process of intestinal and hepatic cholesterol absorption and secretion Adapted from (Park, 2013; Wang, 2007).

Meanwhile HDL, produced both in liver and intestine, recover excessive cholesterol from extra-hepatic tissues. These special lipoproteins have antioxidants, anti-inflammatory and antithrombotic properties. Fully charged HDL return to liver were cholesterol is secreted into bile as cholesterol but also after its metabolization in bile acids, in what is called reverse cholesterol transport (Guay et al., 2012; Ikonen, 2008).

As have been described, intestinal cholesterol uptake is a process mediated by NPC1L1. This transporter is also expressed in the liver, in the bile canalicular membrane of hepatocytes, where it seems to be responsible for the prevention of excessive biliary loss of cholesterol (*Figure 1.1*) (Betters and Yu, 2010). Nevertheless its main expression

occurs in intestine, with a maximum in proximal jejune (Wang, 2007). It is a transmembrane protein with a sterol sensing domain (SSD) localized in the apical membrane of the enterocytes (Betters and Yu, 2010; Hui et al., 2008).This SSD domain is also found in other proteins involved in cholesterol metabolism, namely SREBPs, SREBP cleavage activating protein (SCAP), as well as in HMGCR. Evidence suggests that this domain functions as a binding site to cholesterol modelling sub-cellular localization of proteins containing it (Betters and Yu, 2010).

In particular, the elimination of cholesterol from cultured cells causes translocation of NPC1L1 from the endocytic recycling compartment to the apical plasma membrane subdomain; when cholesterol is reintroduced NPC1L1 is internalized, what associates with the absorption of cholesterol (Betters and Yu, 2010; Ikonen, 2008). It has been suggested that the N-terminal region of NPC1L1 recruits free cholesterol to its membrane location creating a microdomain raft-like rich in cholesterol. When cholesterol reaches the threshold detected by SSD in NPC1L1, that region undergoes clathrin-mediated endocytosis, cholesterol is released in endocytic recycling compartment and NPC1L1 can return to the apical membrane (Betters and Yu, 2010). This transporter is the most probable target of ezetimibe, a pharmacological drug used to specifically block intestinal cholesterol absorption. The co-administration of ezetimibe and statins, in order to control development of hypercholesterolemia, has been successful. Ezetimibe results in a decrease of cholesterol esterification and, therefore, in a decrease of cholesterol for assembly into nascent chylomicrons. NPC1L1 inhibition seems to result in beneficial effects on components of MetS, such as NAFLD, obesity and insulin resistance (Betters and Yu, 2010; Hui et al., 2008; Wang, 2007).

The NPC1L1 shares 50 % of the amino acid sequence with Niemann-Pick C1 (NPC1) a glycoprotein which also presents the SSD and functions as an intracellular transporter of cholesterol. It is fundamental to the correct transport of cholesterol from internalized LDL, its malfunction results in cholesterol storage disorders, once the impairment in cholesterol intracellular transport reduces sensitivity of the endoplasmic reticulum to cellular response to cholesterol levels (Afzali et al., 2013; Betters and Yu, 2010; Wang, 2007). Interestingly, the promoter of *NPC1* gene was found more frequently methylated in patients suffering from CVD, resulting in a weaker expression of this transporter. On the other hand, demethylation of *NPC1* promoter contributes to higher HDL levels and to the decrease in total levels of triglycerides, showing not only the importance

of this transporter, but also the influence of epigenetics, that will be discuss later, in health and disease (Afzali et al., 2013).

The essential reverse cholesterol transport to excess cholesterol secretion is dependent on ATP Binding Cassete (ABC) type A1 (ABCA1), which is ubiquitously expressed and mediates the rate-limiting step in the loading of HDL. The ABCA1 transporter promotes phospholipids and non-esterified cholesterol transfer from peripheral cells to apolipoprotein apoA-I an essential step in globular HDL particles maturation which results in increased excretion of these compounds preventing their harmful accumulation in cells and blood vessels (Afzali et al., 2013; Guay et al., 2012; Ikonen, 2008). The *ABCA1* gene is under epigenetic regulation. The levels of promoter methylation was found higher in adults exposed to situations of starvation in the pre-natal state, suggesting that in these cases the action of ABCA1 is diminished. Simon-Pierre and colleagues, in 2012, showed that patients who suffer from coronary artery disease have a higher degree of *ABCA1* promoter methylation when compared to healthy individuals. This condition is related to a decrease in size and the amount of HDL in circulation, reducing protection level (Guay et al., 2012).

Other important ABC transporters are ABC type G5 and G8 (ABCG5/G8) which form a dimer and limit sterol absorption, while also promoting biliary sterol secretion. When expressed alone ABCG5 or ABCG8 serve only as a non-functional half-transporter, which is accumulated in the endoplasmic reticulum. Unlike other carriers of the same family both ABCG5 and ABCG8 present only 6 transmembrane domains (Yu et al., 2002, 2014). These proteins are located both in the apical membrane of the enterocytes, facing the intestinal lumen but also in the canalicular membranes of hepatocytes, where they promote hepatic cholesterol secretion into bile, (*Figure 1.1*). There is a negative correlation between the cholesterol absorption rate and the expression level of AGCG5/ABCG8 in jejunum and ileum but not in duodenum, the main site of cholesterol absorption (Betters and Yu, 2010; Hui et al., 2008; Wang, 2007; Yu et al., 2002). The ABCG5/G8 dimer is determinant to control sitosterols (plant sterols) absorption, since a genetic defect in *AGCG5/G8* genes results in sitosterolemia, a disease characterized by excessive sitosterol accumulation (Yu et al., 2002). This explains why the process of sterol absorption is a selective process, in which plant sterols, as well as various other sterols, are poorly absorbed or not absorbed at all (Wang, 2007). Apparently, a high *ABCG5* and *ABCG8* gene expression results in an increment of gene expression related

to cholesterol synthesis, without influences in mRNA levels of *SREBP-1c* and *-2*, *CYP7A1*, *LDL receptor* and *ABCA1* (Yu et al., 2002).

1.2.2 - Sterol Regulatory Element-Binding Proteins

The transcription factors SREBP are master regulators of lipid homeostasis, controlling the gene expression of proteins engaged in endogenous cholesterol, FA, triacylglycerol and phospholipid metabolism (Eberlé et al., 2004; Ruiz et al., 2014). There are three known isoforms of SREBP: SREBP-1a, -1c and 2. SERBP-1a and -1c are result of alternative splicing of start site of gene transcription on 17q11,2 human chromosome, while SREBP-2 is codified by a single gene on 22q13 human chromosome. All of them need to be proteolytic activated, as described later. The structural difference between isoforms -1a and -1c is a longer acidic transactivation segment in SREBP-1a, which increases the transcriptional activation capacity (Hashimoto et al., 2006). Regarding their expression, *SREBP-1a* is predominant in cultured cell lines and highly proliferative tissues, such as spleen and intestine, whereas *SREBP-1c* is prevalent in liver, white adipose tissue, skeletal muscle, adrenal gland and brain, both in mice and humans (Eberlé et al., 2004; Horton et al., 2002).

The isoform SREBP-1a is a strong activator of all genes regulated by the family of these transcription factors: those implicated in cholesterol, FA and triglycerides synthesis and also in glucose utilization (Hashimoto et al., 2006; Horton et al., 2002; Ruiz et al., 2014; Weber et al., 2004).

Conversely SREBP-1c is more specific and does not activate cholesterol synthesis. SREBP-1c action is guided to transcription of genes such as those codifying ATP citrate lyase, necessary to cytoplasmic acetyl-CoA production, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), involved in palmitate production. Other targets are the rate-limiting enzyme of FA elongase complex, which converts palmitate to stearate by incorporation of two additional carbons, and stearoyl-CoA desaturase, which inserts unsaturation in stearate to oleate. Additionally, SREBP-1c is also responsible for transcriptional activation of the first enzyme in triglyceride and phospholipid synthesis mitochondrial glycerol-3-phosphate acyltransferase and, it upregulates the glycolytic L-pyruvate kinase, which catalysis both pyruvate and ATP production (Horton et al., 2002; Ruiz et al., 2014).

SREBP-2, expressed in most cell lines and tissues, predominantly in the liver, promotes gene transcription of proteins involved in cholesterol endogenous biosynthesis,

namely, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase (HMGCS) and the rate limiting enzyme of cholesterol synthesis HMGCR, also farnesyl diphosphate synthase and squalene synthase. SREBP-2 also regulates the expression of the *LDL receptor*, which mediates cholesterol uptake through internalization of serum LDL (Horton et al., 2002; Li et al., 2013a).

The SREBPs are membrane proteins whose localization depends on cellular sterol concentration. A very small quantity (about 1 % of total) of cellular cholesterol is contained in endoplasmic reticulum membranes, cholesterol levels in the endoplasmic reticulum modulate SREBPs activation. Once SREBP is produced in endoplasmic reticulum, it associates with another protein, SCAP, that contains a conserved sterol-sensing domain (SSD), also found in other cholesterol related proteins (Ikonen, 2008). When cholesterol is high, it binds to SSD of SCAP and an auto-oxidized form of cholesterol (25-hydroxycholesterol) binds to a chaperone anchored in endoplasmic reticulum, the insulin induced gene (INSIG). The simultaneous binding of cholesterol and its oxidized product promotes INSIG-SCAP-SREBP association in order to block the reticulum to Golgi complex transport of SREBP. On the other hand, the transport of SCAP-SREBP complex to Golgi complex is enhanced when cholesterol levels drop (Ikonen, 2008; Weber et al., 2004). There, proteolytic processing occurs by two functionally distinct steroid sensitive proteases, site 1 protease (S1P) and site 2 protease (S2P), cleaving the hydrophilic luminal loop and the first transmembrane fragment, respectively, releasing the N-terminal fragment with 68 kDa, the nuclear form of SREBPs in the cytoplasm, that translocate to nucleus, as represented in *Figure 1.2* (Li et al., 2013a).

The regulation before the proteolytic process is not the same for the SREBP-1c isoform (Hegarty et al., 2005). The -1c isoform, *in vivo*, seems to be regulated through nutritional status variations and not by sterol depletion. Insulin and LXRα have a positive effect, in SREBP-1c signalling, unlike glucagon (Eberlé et al., 2004; Horton et al., 2002). A study conducted in 2005 by Hegarty and co-workers using isolated primary hepatocytes showed that insulin lead to a marked accumulation of SREBP-1c nuclear form due to an increase in precursor cleavage (Hegarty et al., 2005). This result could be explained by evidences that showed the specific action of insulin in INSIG isoforms.

There are three different isoforms of INSIG: *INSIG-1*, and isogenic *INSIG-2a* and *-2b*. The expression of *INSIG-1* seems dependent on SREBP-2 activation. *INSIG-2b* is ubiquitously expressed and *INSIG-2a* is mainly expressed in the liver. Insulin action seems to be mediated by mRNA down-regulation of *INSIG-2a* without affecting *INSIG-*

1, selectively allowing the endoplasmic reticulum to Golgi transport and consequent cleavage of SREBP-1c, as represented in *Figure 1.2*. (Ferré and Foufelle, 2007; Yabe et al., 2003). LXR induces both *SREBP-1c* and *INSIG-2* expression, probably as a preventive mechanism, in the presence of oxidized derivatives of cholesterol (LXR agonists), as described later (Hashimoto et al., 2006; Hegarty et al., 2005).

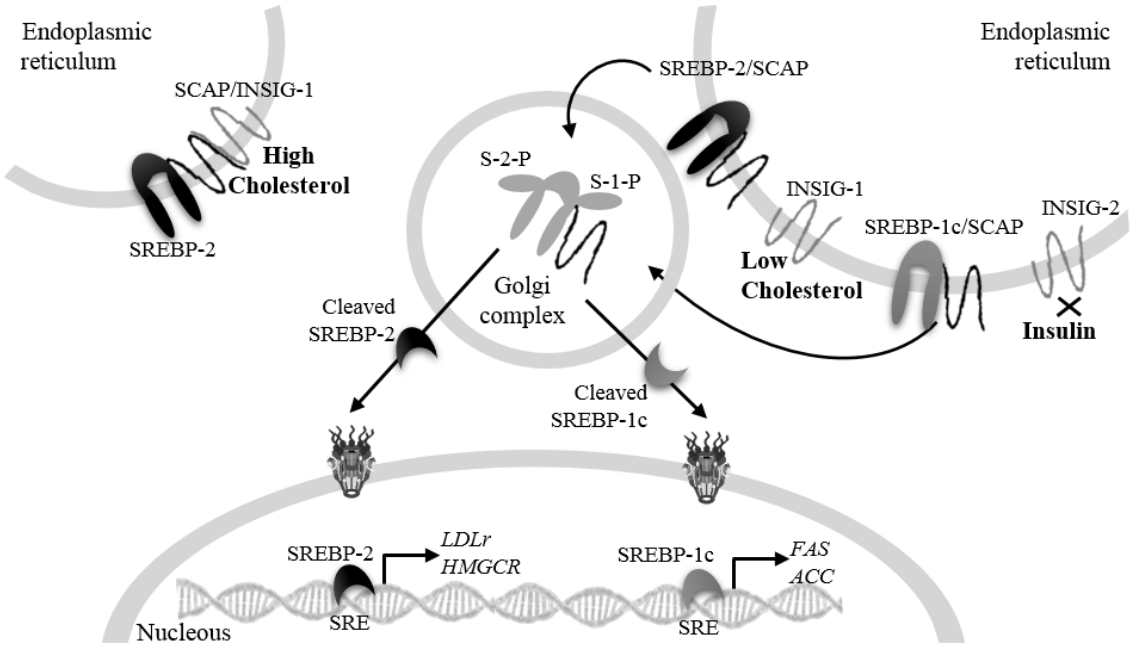


Figure 1.2 – Explanatory scheme of SREBP-2 and SREBP-1c transcription factor activation in response to cholesterol and insulin levels, respectively.

SREBP metabolism regulation can also occur through epigenetic mechanisms involving microRNAs, which are concomitantly expressed. The evolutionarily conserved microRNA miR-33 is presented as two intronic versions: miR33a and miR33b, originated through intron 16 of SREBP-2 and SREBP-1, respectively (Allen et al., 2012; Rotllan and Fernández-Hernando, 2012). The inhibition targets of both miR-33a and b have different functions, such as FA β-oxidation [through carnitine palmitoyltransferase 1 (CPT1)], carnitine O-octanoyltransferase and 5-adenosine monophosphate-activated protein kinase (AMPK).

The transporter ABCA1, in basolateral membrane of hepatocytes, and both ABCG11 and ATPase aminophospholipid transporter 8B1 (ATP8B1), at the apical membrane, involved in sterol efflux are inhibited by miR-33. Also, the ABCG1 and NPC1, which, respectively, mobilizes cellular free cholesterol to HDL particles and moves cholesterol from lysosomes to other cellular compartments, are targets of miR-33a and -b (Allen et al., 2012; Rayner et al., 2010; Rotllan and Fernández-Hernando, 2012). Thereby,

miR-33 limits cholesterol and other lipids secretion, while SREBP-2 promotes their synthesis, both acting synergistically. Studies have shown that anti-miR-33 strategies lead to an increased of sterols secretion in bile as well as improve reverse cholesterol transport since that ABCA1 production is no longer inhibited, an effect that should increase reverse cholesterol transport into new nascent HDL, increasing their levels (Allen et al., 2012).

Other microRNAs, which have been associated with post-transcriptional regulation of genes involved in lipid metabolism are miR-122, miR-370, miR-758 and miR-128-2. miR-122 is the most predominant in liver, representing about 70 % of hepatic microRNAs. Its expression seems to be responsible for a down-regulation of FA β-oxidation and, on the other hand, for an increase in cholesterol synthesis, reflected in increased LDL and HDL levels (Rotllan and Fernández-Hernando, 2012). MicroRNA miR-370 seems to up-regulate miR-122, resulting in an indirect action on the promotion of lipogenesis and inhibition of β-oxidation. Directly, miR-370 down-regulates CPT1 (Iliopoulos et al., 2010). The expression of miR-758 decreases when cholesterol concentration rises, leading to a greater expression of *ABCA1* gene avoiding cholesterol accumulation (Rotllan and Fernández-Hernando, 2012). miR-128-2 has a recognized pro-apoptotic function, but was recently associated with ABCA1 and ABCG1 down-regulation, therefore, limiting cholesterol reverse transport. In addition, it seems to directly reduce the expression of LXR (Adlakha et al., 2013).

1.2.3 - Liver X Receptor and Farnesoid X Receptor

The ligand based nuclear receptor LXR and FXR are also deeply involved in the regulation of lipid metabolism (Fuchs, 2012; Jakobsson et al., 2012). LXR responds to changes in the nutritional state, namely its activation occurs in the presence of oxysterols, while it is inhibited by polyunsaturated FA. Its activation promotes gene transcription related to cholesterol homeostasis, lipogenesis and reverse cholesterol transport, favouring bile sterol excretion (Chen et al., 2004; Miao et al., 2004). On the other hand, FXR is activated by bile acids endorsing the transcription of genes associated with bile acid metabolism, inhibiting their synthesis (Ding et al., 2014). Thus the action of these two nuclear receptors has to be coordinated.

There are two different isoforms of LXR: LXRα and LXRβ which share about 80 % of their binding domains to DNA and ligands. What makes them different is their specific expression, LXRα is expressed in liver, intestine, adipose tissue, macrophages, kidney and gonads, while LXRβ has an ubiquitous expression.

These nuclear receptors bind to DNA upon association with retinoid X receptor (RXR), in a specific consensus sequence LXR responsive element (LXRE), as represented in *Figure 1.3* (Boussac et al., 2013; Jakobsson et al., 2012; Miao et al., 2004; Zhao and Dahlman-Wright, 2010).

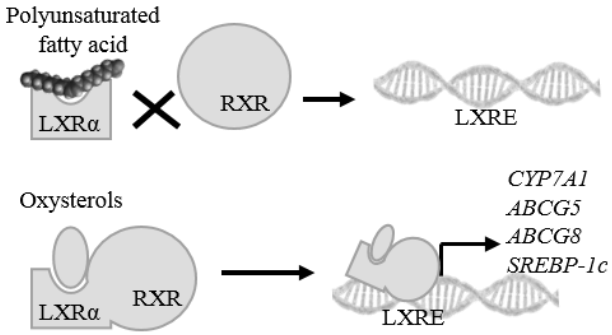


Figure 1.3 – Representation of gene expression control through LXR/RXR.

This specific sequence is found in promoter regions of *CYP7A1*, *ABCG1*, *FAS* and *SREBP-1* genes, among others. In fact, the transcription of the gene that codifies CYP7A1 is stimulated by LXRα, thus inducing bile acid synthesis. Also, LXRα induces *ABCG5/G8* gene expression promoting non-esterified cholesterol elimination, counteracting the increase of dietary cholesterol. In agreement, *NPC1L1* expression drops due to LXR action, increasing the amount of cholesterol absorbed. In addition, *ABCA1* and *ABCG1* in macrophages, involved in HDL assembly and loading, are also stimulated (Araki et al., 2012; Repa et al., 2002; Wang, 2003; Yu et al., 2002; Zhao and Dahlman-Wright, 2010). It has been suggested that LXR negatively regulates cholesterol synthesis and, in parallel, activates gene expression of E3 ubiquitin ligase inducible degrader of LDL receptors, reducing the intracellular pool of cholesterol (Boussac et al., 2013). The LXR receptor plays a role relating dietary cholesterol to FA synthesis, since gene expression of *SREBP-1c*, but not *SREBP-1a* and *-2*, is specifically promoted through LXR due to the presence of cholesterol derivatives. The specificity of LXR may be due to LXRE found only in *SREBP-1c* proximal promoter (Repa et al., 2002). Thus, LXR induces *SREBP-1c* expression and insulin is the main responsible for SREBP-1c activating cleavage, consequently resulting in induced *FAS* and *ACC* transcription. FAS catalyses the last steps of FA biosynthesis while ACC, the rate-limiting enzyme of FA synthesis, constitutes a regulatory bridge between FA synthesis and FA β-oxidation. In the active non-phosphorylated state ACC synthetises malonyl-CoA that is then consumed by FAS activity. However, malonyl-CoA inhibits FA β-oxidation through CPT1 inhibition, leading to FA accumulation. The inhibition of ACC, through phosphorylation by AMPK, results in

both direct inhibition of FA synthesis and indirect FA β-oxidation activation through decreased malonyl-CoA production, as represented in *Figure 1.4* (Koo, 2013).

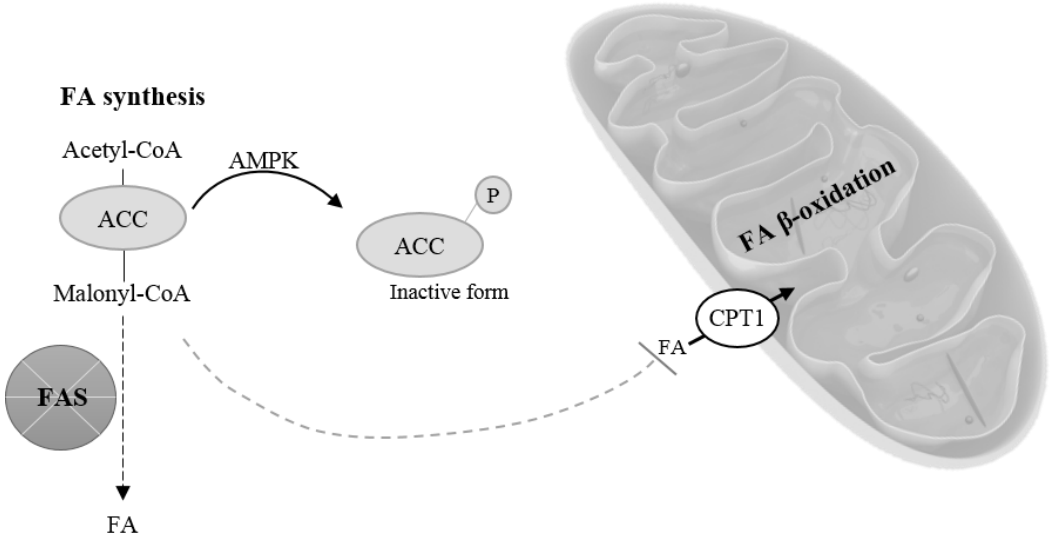


Figure 1.4 – Regulatory interplay between synthesis of fatty acid in cytosol, and fatty acid β-oxidation in mitochondria.

Thus SREBP-1c is an important regulator of FA synthesis and FA β-oxidation balance (Koo, 2013). Its induction due to LXR could result in FA production needed to cholesterol esterification, protecting cells against high free cholesterol concentration (Ferré and Foufelle, 2007; Repa et al., 2002).

FXR is also a ligand-activated transcription factor with high levels of expression in the liver, intestine, kidney, and adrenal cortex, and low levels of expression in the heart, lung and adipose tissue. This transcription factor binds to DNA as a monomer but also as a heterodimer, by concomitant binding to RXR, in FXR responsive element of gene promoters related to bile acid and lipid metabolism (Xu et al., 2014). The activation of FXR occurs in the presence of bile acids and to a lower extension by farnesoid, an intermediate of the mevalonate pathway. FXR indirectly inhibits *CYP7A1* gene expression, decreasing bile acid synthesis. Also FXR stimulates bile acid secretion once it specifically promotes the expression of bile salt export pump and *ABCG5/G8* gene. The removal of triglycerides from circulation could be increased by FXR, since it stimulates lipoprotein lipase and VLDL receptor. In agreement, scavenger receptor class B type I (SR-BI) is up-regulated when FXR is activated resulting in a higher transport of HDL into liver for elimination of cholesterol as bile acids. In addition, FXR blocks SREBP-1c and therefore inhibits FA synthesis, while promoting PPARα and its target CPT1 resulting in an increased rate of

FA β-oxidation. Thus FXR activation by bile acids results in improved reverse cholesterol transcription and FA consumption, reducing both cholesterol and FA accumulation (Baptissart et al., 2013; Fuchs, 2012; Xu et al., 2014). Therefore, FXR agonists could be seen as therapeutic opportunities to NAFLD (Fuchs, 2012).

1.2.4 - Peroxisome Proliferator-Activated Receptor

The peroxisome proliferator-activated receptors are another family of ligand-activated transcription factors. There are three known members: PPARα, PPARγ and PPARβ/δ which have different targets and functions in metabolic regulation. When in the presence of ligands, PPARs bind to RXR forming heterodimers that bind to specific DNA sequences called peroxisome proliferator response element (PPRE) (Tyagi et al., 2011; Usuda and Kanda, 2014). While PPARα (mainly expressed in liver, kidney, heart, muscle and adipose tissue) regulates FA β-oxidation in mitochondria, peroxisomes, and microsomes, PPARγ (namely expressed in adipose tissue) controls triglycerides uptake and storage in adipocytes, therefore, controlling triglycerides levels to undergo hepatic FA β-oxidation, storage as triglycerides or secretion into circulation in VLDL particles. PPARβ/δ (higher expression in brain, adipose tissue and skin), less studied, is also involved in FA metabolism, however, its function seems to involve the reduction of inflammatory mediators gene expression and adhesion molecules, effects that could mitigate atherogenesis (Giby and Ajith, 2014; Gusdon et al., 2014; Tyagi et al., 2011; Usuda and Kanda, 2014).

The transcription factor PPARγ is activated by FA and derivatives, and boost the secretion of adiponectin by adipose tissue, which has anti-hyperglycemic effects. PPARγ activation directs non-esterified FA from liver and skeletal muscle into accumulation into adipose tissue (Giby and Ajith, 2014). PPARγ also targets induction of gene expression of FA transport and binding protein, and glucose transporter 4, increasing FA and glucose uptake in adipocytes. It increases glucokinase and phosphoenolpyruvate carboxykinase (PEPCK) enzyme, involved in gluconeogenesis, and induces LXRα gene expression, in adipocytes. Thus PPARγ activation improves insulin sensitivity and action, and in fact is the pharmacological target of the thiazolidinediones (troglitazone, rosiglitazone and pioglitazone) family of antidiabetic drugs which are also used in NAFLD treatment (albeit with controversial effects) (Gusdon et al., 2014; Tyagi et al., 2011; Usuda and Kanda, 2014).

Natural ligands of PPARα are unsaturated and saturated FA, such as palmitate (from endogenous synthesis or from diet), eicosanoids and long-chain fatty acyl-CoAs. However, FA liberated from adipocytes do not activate PPARα (Lefebvre et al., 2006). This transcription factor not only activates transcription of mitochondrial HMG-CoA synthase, which converts acetyl-CoA molecules into ketone bodies during fasting or diabetes, but also regulates gene expression of acyl-CoA oxidase, L-bifunctional protein and thiolase peroxisomal enzymes that degrade normal-chain FAs. PPARα is involved in cellular uptake of FA, since it specifically upregulates FA translocase, in liver and intestine, but not in skeletal muscle. PPARα is also involved in the regulation of FA transport protein stimulating cellular uptake (Lefebvre et al., 2006).

As major regulator of FA β-oxidation process, PPARα controls gene expression of its key enzymes, namely acyl-CoA synthetase, very-long- and medium-chain acyl-CoA dehydrogenases, 3-ketoacyl-CoA thiolase and, specially, CPT1 (Lefebvre et al., 2006). Regulation of CPT1 is fundamental to control FA β-oxidation rate, once that its initiation is dependent on fatty acyl-CoAs convertion to fatty acyl-carnitines by CPT1 in order to achieve their translocation through mitochondrial outer membrane into the intermembrane space and then, by carnitine acylcarnitine translocase, transported though mitochondrial inner membrane. There CPT2 catalyses fatty acylcarnities conversion to fatty acyl-CoAs which undergo β-oxidation in the mitochondrial matrix. Thus CPTs action is crucial for β-oxidation, preventing FA tissue accumulation (Koo, 2013).

PPARα activation has been associated to lower levels of plasma triglycerides by reducing triglycerides available to VLDL secretion due to lipoprotein lipase up-regulation. Moreover, up-regulation of PPARα seems to reduce TNF-α ameliorating inflammatory state in NAFLD patients. In fact, these patients, as well as patients with obesity and related insulin resistance have apparently decreased levels of PPARα (Giby and Ajith, 2014; Gusdon et al., 2014). Fibrates are the most important class of drugs which act as PPARα agonists. Fibrates are safe drugs with lipid lowering properties that shows PPARα agonists as a therapeutic target in MetS and related diseases treatment (Gusdon et al., 2014; Lefebvre et al., 2006).

1.3 - Dietary Changes

There is not an ideal diet, the major guidelines are a varied diet, with consumption of fruits, vegetables and whole grain cereals and low saturated fat intake, behaviours that

have been associated with a lower risk of developing chronic diseases, such as CVD (Liu, 2004).

Over all, diets to lose weight should create an energy deficit. However, the benefits of a specific diet depend on the long-term sustainability of the diet itself and adherence to it (Makris and Foster, 2011). There is a great number of different diets aiming weight loss, as primary care recommended by IDF. As have been said, the change of dietary components can be a useful tool to control certain diseases. One of the most controversial components of diet is fat, although a decrease in T2DM and CVD risk factors, as well as weight loss, have been attained with diets ranging from 10 % fat (low-fat diet) to 45 % fat (moderate-fat diet). That is because a high consumption of vegetables, legumes, fruits and wholegrain cereal, in people physically active, seems to compensate fat intake (Makris and Foster, 2011; WHO/FAO, 2003).

Diets with about 10 % of calories from carbohydrate and approximately 60 % from fat – low-carbohydrate diets – are considered as equally effective in reducing weigh as low-fat diet. However, low-carbohydrate diets have been shown to be additionally effective in decreasing triacylglycerides and VLDL and rising HDL levels. High-protein diets have been seen as particularly effective in reducing fat mass and triacylglycerides, and capable of decreasing waist circumference and intra-abdominal adipose tissue, and consequently improving body composition. These effects seem to be more marked in individuals with dyslipidaemia and in risk for T2DM (Makris and Foster, 2011). Also, low-carbohydrate and high-protein diets seem to prevent cancer development and progression in mice (Ho et al., 2011).

1.3.1 - The Mediterranean Diet Approach

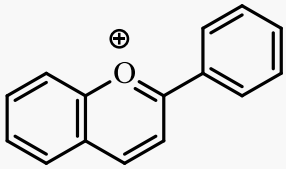
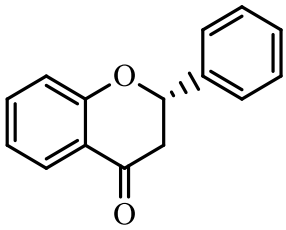
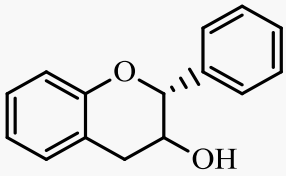
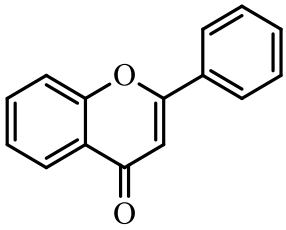
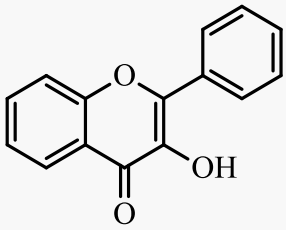
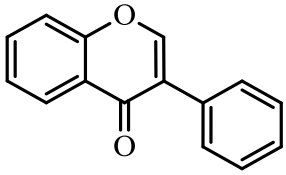
The Mediterranean diet is one of the most studied diet, and is considered a healthy diet in spite of providing at least 30 % of calories as fat – moderate-fat diet. The problem of fat consumption lies in its sources. In the Mediterranean diet, olive oil is the main source of fat, being rich in unsaturated fat and contributing to a high consumption of monounsaturated FA. In addition, the Mediterranean diet is complemented with a great intake of fruits, vegetables, whole grains, legumes, nuts and fiber as well as moderate alcohol consumption. Mediterranean diet favours fish intake, a good source of polyunsaturated FA, such as omega-3 fatty acids and includes low amounts of saturated fat from animal sources (Dilis et al., 2007; Makris and Foster, 2011; Widmer et al., 2014).

One of the benefits of the Mediterranean diet is the fact that it is not restrictive, maintaining nutritional balance, which improves sustainable engagement to this diet. The Mediterranean eating pattern has been correlated with a lower CVD incidence. One of the first evidences of this fact was achieved in the fifties with 6 cohort studies done in 7 countries (Finland, Greece, Italy Japan, the Netherlands, USA and former Yugoslavia) (Assmann et al., 2014). This study was able to show a significant association between diet and incidence and severity of Coronary Heart Disease (CHD). Specifically, the percentage of calories derived from saturated fat were associated with higher CHD, whereas the consumption of monounsaturated fats were related with lower events of CHD, showing the importance of the type of fat consumed (Assmann et al., 2014). More recent, in 2013, Estruch and colleagues, involving the response of 7447 persons from Spain with CVD risk to different diets: a Mediterranean diet supplemented with extra-virgin olive oil, a Mediterranean diet supplemented with mixed nuts, or a control diet (advice to reduce dietary fat). This clinical investigation demonstrated a relative risk reduction of approximately 30 % in both supplemented Mediterranean diets, supporting the benefits related to Mediterranean diet for the primary prevention of cardiovascular disease. Although the control group was advised to maintain a healthy diet and reduce fat intake that was not achieved. Therefore the main differences were the distribution of fat types achieved with the extra-virgin olive oil and nuts supplement. Once again showing the relevance of the type of fat consumed and the validity of Mediterranean diet as a primary strategy to prevent CVD (Estruch et al., 2013).

1.3.2 - Dietary Flavonoids, Specifically Flavones

Another important feature of Mediterranean and Mediterranean-based diets is the high prevalence of fruit and vegetable consumption, which leads to a high flavonoids intake (Dilis et al., 2007). Flavonoids are a large group of natural compounds that are thought to have health-promoting properties, often attributed to their antioxidant properties (Kumar and Pandey, 2013). In plants, flavonoids are synthesized by phenylpropanoid pathway, and chemically, flavonoids consist in polyphenols having a fifteen-carbon skeleton comprising two benzene rings linked by a pyran ring. Their activities are structure dependent. (Kumar and Pandey, 2013; Mehta et al., 2010). Flavonoids can be divided according to the degree of oxidation of the oxygen heterocycle and the substitution patterns in 6 main sub classes – Anthocyanins, Flavanones, Flavan-3-ols, Flavones, Flavonols and Isoflavones – as described in *Table 1.2*.

Table 1.2 – Flavonoids groups, structures, sources and example of specific compounds. Information retrieved from (Egert and Rimbach, 2011; Kumar and Pandey, 2013).

Flavonoids Group	***Structural backbone***	***Compounds***	***Food Sources***
Anthocyanins		Cyaniding; Malvidin; Pelargonidin; Peonidin;	Berries; Aubergine; Black currant;
Flavanones		Eriodictyol; Hesperidin; Naringenin;	Orange; Grapefruit; Lemon;
Flavan-3-ols		Catechins; Epicatechin; Epigallocatechin; Gallocatechin;	Chocolate; Green tea; Beans; Cherry;
Flavones		Luteonin; Epigenin; Baicalein; Chrisin; Nobiletin;	Parsley; Celery; Capsicum pepper;
Flavonols		Kaempferol; Myricetin; Quercetin; Rutin;	Onions; Apples; Curly kale; Leek;
Isoflavones		Daidzein; Genistein; Glycitein;	Soy flour; Soybeans; Soymilk;

Flavonoids has been considered cancer preventive components in our diet. Flavonoids consumption also seems to prevent CVD and premature aging, and in fact the Mediterranean populations have lower incidence of CVD (Amic et al., 2007). The antioxidant activity is the most associated to flavonoids, however also anti-inflamatory, antiviral and

hepatoprotective activities are related to this group of compounds (Amic et al., 2007; Kumar and Pandey, 2013; Nijveldt et al., 2001).

To achieve sufficiently active flavonoid concentrations a regular intake of the main sources seems necessary, an occasional dose could not explain healthy benefits attributed to these compounds (Nijveldt et al., 2001). In a research conducted by Vardis Dilis and associates based in traditional Mediterranean diet, where during a week, all meals were strictly prepared and flavonoids intake calculated, especially flavone, flavonol and flavan-3-ol. On average, the daily intake was about 79 mg, of which about 10 mg/day were flavones. The flavones, flavonols, flavan-3-ols and flavanones intake in Mediterranean diets is the highest when compared to northern European and American diets. In particular the flavone Luteolin (L), despite its fluctuating values over a week represents more than 50 mg ingested (Dilis et al., 2007).

A study conducted with Spanish data from European Prospective Investigation into Cancer and Nutrition study Spanish cohort, through diet history assessed by interviews estimate about 313 mg/day intake of total flavonoids (Zamora-Ros et al., 2010). In a more recent work, achieved by McCullough and collaborators, the estimated total flavonoids intake varied between 201 and 268 mg/day for man and woman. This estimative was made using information from questionnaire of participants in Cancer Prevention Study II Nutrition Cohort in United States. These studies showed a protective effect of total flavonoid intake, and in woman the strongest protective correlation was observed with flavones. Note that these associations were attenuated with adjustment for physical activity (McCullough et al., 2012).

Several individual flavones have been associated with health promoting effects. For instance Baicalin, a naturally occurring flavone, seems to prevent MetS in high-fat fed rats, through stimulation of β-oxidation and reduction of *SREBP-1c* gene expression, presenting also beneficial effects againts insulin resistance (Pu et al., 2012). Nobiletin, another flavone compound that is polymethoxylated, also seems able to modulate lipid metabolism in high-fat diet rats (Lee et al., 2013). Both Chrysin and L were found to present insulin resistance modulators activity, mainly because they seem to activate PPARγ, reducing associated hypertensive and vascular complications (El-Bassossy et al., 2013).

Luteolin is one of the most common flavonoids present in edible plants and families of plants used in traditional medicine such as the genus *Salvia*. As others flavones, L occurs mainly in a glycosylated form – luteolin-7-O-glucoside (L7G) in celery, green

pepper, perilla leaf, spinach, apple skin, chamomile tea and in *Salvia officinalis*, etc. L in the aglycone form is very common in perrilla seeds (López-Lázaro, 2009; Sá et al., 2009; Shimoi et al., 1998). In plants, glycosylation reduces the auto-oxidation and reactivity of the 7-OH group and increases the solubility of flavones in the aqueous cellular environment, allowing its storage in the cell vacuole (Amic et al., 2007; Kumar and Pandey, 2013). Although the glycosylated form is the most common it seems that glycosides are hydrolyzed by the β-glucosidase enzyme in the gut. The absorption of aglycones is easier occurring in greater quantities and faster (Park and Song, 2013).

Both L and L7G have been associated with a great number of biological activities, such as anti-inflammatory (↓ NFκB), antioxidant (scavenging of reactive species), antimicrobial and anti-cancer activity (↑ wild-type p53), reviewed in (López-Lázaro, 2009). In addition, are related to cardio-protective and antidiabetic properties, once it seems to be capable of control *SREBP-1c* gene expression and related genes such *FAS* and *CPT1*, but also sensitize cells to insulin by PPARγ (Ding et al., 2010; Liu et al., 2014, 2011). Thus, these flavones abundant in Mediterranean diet seem to have potential as natural compounds preventive of MetS and related diseases.

1.4 - Physical Activity

As already mentioned, an important component of a healthy lifestyle is the fight against sedentary and physical inactivity. These behaviors has been identified as the fourth leading risk factor for global mortality (WHO, 2010). To dedicate time to exercise in our lives is a primary recommendation as preventive measure for metabolic related diseases, cancer and for general health maintenance made by World Health Organization, Food and Agricultural Organization and IDF (IDF, 2006; WHO/FAO, 2003; WHO, 2010).

The lack of daily exercise is associated with greater risk of CVD, T2DM and cancer, but also related to worsening other risk factors, such as obesity and high blood pressure, for example (WHO, 2010). On the other hand, a wide prospective study showed that physical activity engagement (vigorous exercise of 20 min ≥ 3 times/week) could be related to a 32 % reduction in mortality risk. It seems that benefits associated to exercise help both normal weight persons and overweight persons even if exercise do not result in weight loss (Leitzmann and Park, 2007). A meta-analysis using 45 published articles, related to lifestyle and cancer survivors, concluded that physical activity seems to reduce

mortality of breast and colon cancer patients. These results were attributed to insulin levels and signaling, inflammation and putatively immunity which may be improved with exercise engagement (Ballard-Barbash et al., 2012).

Evidences suggest that physical activity has beneficial effects in control of disorders related with lipid metabolism. Physical exercise leads to improvement in lipid profiles rising HDL levels and decreasing LDL, VLDL, total cholesterol and triglyceride levels (Halverstadt et al., 2007; Meissner et al., 2010). These result may be from an improvement in reverse cholesterol transport (Meissner et al., 2010; Moreira et al., 2013). A study by Meissner and co-workers using mice subjected to voluntary exercise in wheel, suggested that physical activity during 2 weeks increased fecal neutral sterol and bile acid excretion. Also a decreased cholesterol absorption (NPC1L1 lower mRNA levels was observed) but an increase in its endogenous synthesis (HMGCR higher mRNA levels was simultaneously registered) (Meissner et al., 2010).

Physical exercise, besides improving lipoproteins levels, also causes changes in their relative proportions and sizes, namely increases the number of large HDL's, which is a cardiovascular protective factor. The study by Halverstadt and associates, included over one hundred sedentary volunteers and indicated positive effects on the composition and number of lipoproteins, after 24 weeks of supervised endurance exercise (3 sessions/week, 1 h).These benefits which are not totally dependent on diet, initial body composition and weight reduction over training (Halverstadt et al., 2007).

Beneficial effects of exercise are also associated with NAFLD prevention and treatment. In an investigation with adults in Nonalcoholic Steatohepatitis Clinical Research Network data, where physical activity was auto-reported, the severity of disease was analyzed according to the type of exercise activity: sedentary, moderate and vigorous. The results showed that only vigorous exercise decreased the progression to more severe states, it was, however, not possible to determine the optimal duration and intensity of exercise directed to NAFLD prevention or treatment (Kistler et al., 2011).

The effects of physical activity on health maintenance and prevention or treatment of diseases, may result from alterations of gene expression due to epigenetic mechanisms, this is exercise may alter our epigenome in a beneficial way (Tzanninis et al., 2013). Which also applies to Mediterranean diet (Ling and Rönn, 2014).

1.5 - Epigenetic Regulation of Gene Expression

The term epigenetic was introduced for the first time in the early 1940s by Conrad Waddington a geneticist that used the concept to relate the interaction of genotype with environmental factors (Jablonka and Lamm, 2012). After, Arthur Riggs and associates understood epigenetic as changes in gene function mitotically and/or meiotically heritable that are not explained by changes in DNA sequence, so is not inheritance of mutations (Bird, 2007). In the 1970s Holliday and Pugh were the first to propose covalent modifications of DNA, namely methylation of bases, as the mechanism responsible for the fact evidenced for Conrad. This was later confirmed by evidences that genomic imprinting and X-inactivation in mammals are due to epigenetic mechanisms (Dolinoy et al., 2007; Holliday and Pugh, 1975).

Epigenetics explains why the clarification of the genetic code is not enough to fully understand the information encoded by DNA. The study of the genetics of an organism is, therefore, not sufficient to define their susceptibility to a particular condition. The epigenome is substantially more dynamic, varying during development and cell differentiation in response to environmental factors (Huidobro et al., 2013). In fact, the transposition of the DNA code to the phenotype is dependent on DNA methylation pattern, histone modifications, non-coding RNAs and other mechanisms of chromatin remodelling that together control how DNA is packaged in chromatin and, therefore, how accessible it is to transcription. All these mechanism of regulation are epigenetic marks that can be communicated to the cell lineage and across generations, representing common patterns of epigenetic inheritance (Huidobro et al., 2013; Kirchner et al., 2012). Epigenetic is today viewed as the study of heritable changes in gene expression, without alterations in the nucleotide sequence, involving methylation of DNA sequence and histone post transcriptional modifications that leads to chromatin remodelling, as well as microRNAs (Wegner et al., 2014).

The epigenetic mechanisms most studied are DNA methylation and post-translational histone modifications, two independent processes which can act together synergistically (Dolinoy et al., 2007; Kirchner et al., 2012). Histones are structural proteins responsible for stabilization and packaging of DNA. The N-terminal of histones can undergo acetylation, methylation, phosphorylation, ubiquitination, glycosylation and poly-ADP ribosylation in a dynamic and reversible process. These post-translational changes affect histone affinity to DNA and of DNA to histones therefore affecting the level of

chromatin compression hence of gene expression (Huidobro et al., 2013; Roth et al., 2001). Acetylation is catalysed by histone acetyltransferases (HAT), in lysine residues, using as acetyl groups donors the acetyl CoA, generally resulting in loss of positive charge and therefore diminishing DNA packaging. Thus an enrichment in acetylated histones are typical in transcriptionally active sequences. The acetylation could occur immediately after histone production, in cytoplasm, or in the nucleus to promote transcription. The enzymes which remove acetyl groups are histone deacetylases (HDAC) (Huidobro et al., 2013; Kirchner et al., 2012; Roth et al., 2001). Another important histone modification is methylation, which leads to alterations in transcription levels, according to the residue but also extension of methylation. The result could be either activation as inhibition of transcription (Huidobro et al., 2013; Kirchner et al., 2012).

In mammals, the percentage of nucleic acids methylation process of nucleic acids is known to occur in cytosine nucleotides, typically in cytosines that precede guanines – dinucleotide CpG. The methyl group is transferred to 5-carbon in cytosine pyrimidine ring from S-adenosylmethionine (SAM) releasing S-adenosylhomocysteine (SAH), as shown *Figure 1.5* (Dolinoy et al., 2007; Huidobro et al., 2013; Jones and Baylin, 2002; Kirchner et al., 2012). Methyl donors are nutrients obtained from dietary methionine, choline and folate. It's absence influences SAM levels and influences DNA methylation patterns (Varela-Rey et al., 2013). The methylation of DNA may cause gene silencing once it can inhibit the binding of transcription factors whose recognition sites are rich in CpG (Ho and Tang, 2007).

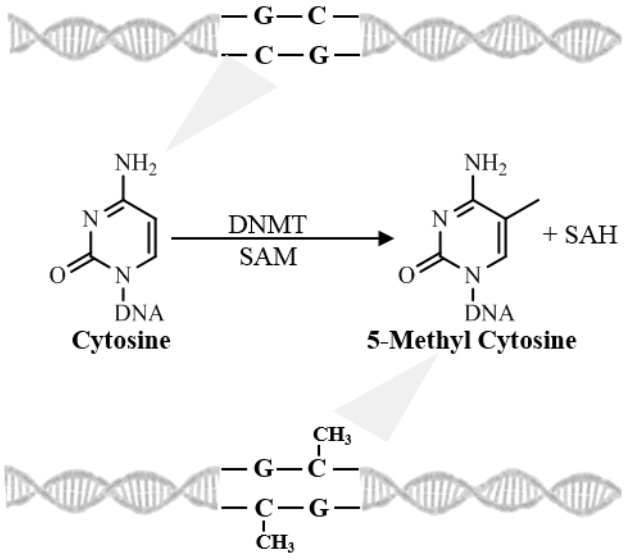


Figure 1.5 – DNA methylation of CpG dinucleotide, by DNMTs. Retrieved and adapted from (May, 2010).

The CpG sequences are not randomly distributed, being preferencially found in CpG islands, small regions between 0.5 and 4 kb, found in about 50 % of human genes, following specific patterns of tissue and cellular differentiation. CpG repetitive sequences, and intergenic regions in the body of the genes are highly methylated, which is related to the maintenance of chromosomal stability, When the promoter regions of the genes are hypermethylated, gene transcription is disabled, in healthy conditions may have to do with genomic imprinting, genes located on the X chromosome in females and tissue specific gene silencing. Abnormal DNA methylation may be responsible for gene silencing and disease conditions (Huidobro et al., 2013; Jones and Baylin, 2002).

DNA methylation is carried out by four different enzymes DNA methyltransferases (DNMT): DNMT1, DNMT3A, DNMT3B and DNMT3L. DNMT1 enzyme is responsible for the maintenance of existing methylation patterns, therefore DNMT1 is generally found in association with the DNA replication machinery, where it is highly specific for hemimethylated CpG dinucleotide produced during DNA synthesis (Bergman and Cedar, 2013). DNMT3A and DNMT3B catalyse *de novo* methylation acting in dinucleotides non-methylated and have no preference for non-methylated or hemimethylated chains. When DNMT3L interacts with DNMT3A and DNMT3B methylation of retrotransposon is facilitated (Calcagno et al., 2013; Huidobro et al., 2013).

Deregulation of epigenetic mechanisms may be prejudicial either by hypermethylation or by hypomethylation, depending on the areas it affects. On the one hand hypomethylation, loss of normal DNA methylation, can cause destabilization of the genetic material and promote the transcription of genes normally suppressed; on the other hand, hypermethylation of gene promoter may lead to transcriptional silencing and could therefore promote the elimination of important proteins to the cells healthy functioning. For example, hypermethylation of tumour suppressor *MLH1*, an enzyme of the mismatch repair complex, causes its inactivation and has been observed in colorectal cancers (Hammoud et al., 2013). Hypermethylation of MLH1, and other mismatch repair mechanism, such as *PMS2* and *MSH2*, were also identified in hepatocellular carcinoma (Hinrichsen et al., 2014).

Over-expression of DNMTs has been identified in hepatocelular carcinome, gastric and colorectal cancer (Calcagno et al., 2013; Jones et al., 2006; Park et al., 2006). The DNMT1 and DNMT3A enzymes are often over-expressed, although over-expression of DNMT3B is more significant, leading to chromosomal instability and cancer. The elimination of DNMT3B enzyme specifically prevents the progression of cancer process in

models of colorectal cancer (Huidobro et al., 2013). In spite of the frequent CpG island hypermethylation, cancer development is associated with a global decreased of DNA methylation – global hypomethylation, (even with vulnerable genes undergoing specific hypermethylation) (Calcagno et al., 2013). A decrease in global DNA methylation also seems to occur with aging, in agreement with increased incidence of various pathologies related to aging, probably due to a progressive reduction of DNMT1 expression, while up-regulation of DNMT3B may be responsible for specific hypermethylation (Lao and Grady, 2011).

As mentioned above, DNA methylation processes are reversible and variable, which is why even in healthy individuals the methylation patterns are different. The epigenome is affected by environmental exposures, gender dependent, and is characteristic of each tissue and age of the individual (Dolinoy et al., 2007; Huidobro et al., 2013). Exercise and diet are two additional factors that have been proposed as modulators of changes in DNA methylation through which they mediate their effects on gene transcription (Nitert et al., 2012). In fact, exercise seems to affect global DNA methylation either be its increase or decrease depending on the tissue. For instance, in adipose tissue, physical activity increase methylation (Rönn et al., 2013), whereas in skeletal tissue, the changes in methylation levels of altered genes is mainly due decrease DNA methylation in response to exercise (Nitert et al., 2012). Diet seems to affect more DNA methylation because is a source of methyl donors essential for methylation process. A high-fat diet seems to have a higher influence in prenatal and early postnatal state on epigenome (Waterland and Rached, 2006). However, the effect of high-fat diet consumption on global DNA methylation, as far as we know was not described.

Chapter 2 - Experimental Part A: Effects of Exercise on Cholesterol Metabolism in High-fat Fed Rats: Involvement of Epigenetic Mechanisms

2.1 - Chapter Introduction

Diet and physical activity are two critical aspects of our lifestyle which condition our health. Diseases associated with abnormal metabolism have been increasing due an increase of sedentary lifestyle combined with a decrease in healthy foods, typical of Western world (Kaur, 2014; WHO/FAO, 2003).

The onset of these chronic diseases can be prevented, but also when these conditions are already established, changes in lifestyle may be beneficial. Namely, healthy diets and increased physical activity, are recommended as therapeutic measures (IDF, 2006). One of the beneficial effects attributed to increased physical activity is the control of lipid metabolism (Halverstadt et al., 2007; Meissner et al., 2010). Even if exercise does not result in weight loss, both normal weight persons and overweight persons benefit from incorporation of physical activity in our daily lives (Leitzmann and Park, 2007). Physiological changes due to physical activity have been explained by changes in expression of genes determinant in biological pathways. These changes have been suggested to be due to alterations in epigenetic marks: such as DNA methylation and histones modifications, which are highly influenced by lifestyle and environmental factors (Tzanninis et al., 2013).

In a previous study, aiming to assess the preventive effect of voluntary physical activity and the potential therapeutic effect of endurance training it was used a model of obese rats with associated metabolic disorders, induced by standard and high-fat Lieber-DeCarli diets. It seems that only endurance training was able to attenuate obesity related measures and histological features of NASH, counteracting NAFLD state progression (Gonçalves et al., 2014a, 2014b). Since that these two components of lifestyle may alter epigenetic mechanism and they may also influence cholesterol metabolism at colon level, our aim, was to analyse differences in cholesterol absorption and excretion mechanisms, in colon samples, in response to the different diets (standard vs high-fat) and the different exercise regimen (voluntary or endurance training vs sedentary lifestyle). We also evaluate epigenetic changes in global DNA methylation

2.2 - Material and Methods

Reagents, antibodies and primers

TRI Reagent used for DNA and RNA simultaneous isolation was purchased to Sigma-Aldrich (St. Louis, MO, USA), as was bovine serum albumin (BSA), Formamide,

NP-40, 3-(N-Morpholino) propanesulfonic acid (MOPS), Triton X-100 and Tween-20. Formaldehyde used in the denaturing agarose gel was acquired from José Vaz Pereira, Lda. (Sintra, Portugal). While agarose was purchased from NZYTech, Lda. (Lisboa, Portugal). The RNA stain Safe-Green™ to was acquired from Applied Biological Materials Inc. (Richmond, BC, Canada). Sodium deoxycholate used in lysis buffer was purchased to Fluka/BioChemika (Buchs, Switzerland), and glycerol was purchased to Panreac Quimica SA (Barcelona, Spain).

The imprint® Methylated DNA Quantification Kit used in evaluation of global DNA methylation was acquired from Sigma-Aldrich (St. Louis, MO, USA). The DC protein assay kit and Clarity™ Western ECL Substrate for chemiluminescent detection, as well as iScriptTM cDNA Synthesis Kit and SsoFast EvaGreen supermix, utilized in quantitative real-time PCR were acquired from Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All other reagents were of analytical grade.

The antibody against β-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA). The anti-mouse secondary antibody containing IgG horseradish peroxidase was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).The primers used in qRT-PCR are indicated in *Table 2.1*. Primer sequences were either found in literature or designed by ourselves, and their specificity confirmed using Primer-Blast tool provided by the National Centre for Biotechnology Information (NCBI). Selected primers were manufactured by STAB VIDA, Lda, (Caparica, Portugal).

Table 2.1 – Primers sequences used in qRT-PCR.

Gene	***Primers Sequence***	***NCBI Reference Sequence:***	***Ref.***
SREBP-2	F: ATTCCCTTGTTTTGACCACGC R: TGTCCGCCTCTCTCCTTCTTTG	NM_001033694.1	(Morral et al., 2007)
ABCA1	F: ATCTCATAGTATGGAAGAATGTGAAGCT R: GTACAACTATTGTATAACCATCTCCAAA	NM_178095.2	(Racine et al., 2010)
ABCG5	F: CCCTGTCCTGAACATTCCAA R: TTGGGTGTCCACCGATGTCA	NM_053754.2	(Repa et al., 2002)
ABCG8	F: CGGCTGGCTTCATGATAAAC R: GGAACGACATCTTGGAAATCC	NM_130414.2	(Repa et al., 2002)
NPC1L1	F: GGACCAGATGTTAACCAAGCTC R: AAGCATGGACAAGCTCCTACTC	NM_001002025.1	Designed
β-actin	F: AGAGGGAAATCGTGCGTGAC R: CAATAGTGATGACCTGGCCGT	NM_031144.3	(Hoque et al., 2007)

Colon samples

Colon samples were kindly provided by Prof. Dr. António Ascensão and Prof. Dr. José Magalhães, from Research Center in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto. The colon samples were collected from six different groups of male *Sprague-Dawley* rats, submitted to 2 experimental diets and 2 exercise regimens. The animals were allocated according to diet and type of exercise. Per diet**,** animals were divided between standard and high-fat Lieber-DeCarli diets, both liquid diets, iso- and hypercaloric, respectively, containing the same micronutrients. While standard diet was composed for 35 % energy derived from fat, 47 % from carbohydrates and 18 % from protein, high-fat diet was composed for 71 % from fat, 11 % from carbohydrates and 18 % from protein. This high-fat diet leads to NASH development, reproducing key features of the human pathology (Lieber et al., 2004). A complete description of the diets is presented in supplementary information (*Table S.1*). Rats were initially divided between sedentary group and voluntary group – with free access to a running wheel, for both diet regimens. After 8 weeks, half of the animals in the sedentary groups of the two dietary interventions were assigned to the corresponding endurance training groups. Firstly, for one week, rats were familiarized with the treadmill. After that, animals were submitted to eight weeks of 60 min/day, 5 days/week, at increasing speed, from 15 to 25 m/min, in the treadmill. The other animals maintained the same exercise regimen. As described in (Gonçalves et al., 2014a) in *Figure 2.1* is represented the group identification of rats: standard diet and sedentary (SS group); standard diet and voluntary physical activity (SV group); standard diet and endurance training (ST group); high-fat diet and sedentary (HS group); high-fat diet and voluntary physical activity (HV group); high-fat diet and endurance training (group HT). At the end of the experiment, that lasted 17 weeks, animals were anesthetized (Ketamine 90 mg/kg and Xylazine 10 mg/kg), colon was excised and stored in TRI Reagent at -80 °C.

Total RNA and DNA extraction

Total RNA and DNA were isolated from frozen colon samples using TRI Reagent (Sigma) following the manufacturer's instructions. Briefly 70-100 mg of tissue was cut into small pieces, homogenized using a tissue homogenizer (DREMEL® Multi, Mod. 395) with 0.7-1 mL Trizol. The samples were then centrifuged and left to rest for 5 min at room temperature, after 140-200 μL chloroform were added and the samples were centrifuged

again. Three layers were thus formed: RNA on top (aqueous), DNA in interphase and in the bottom (organic phase).

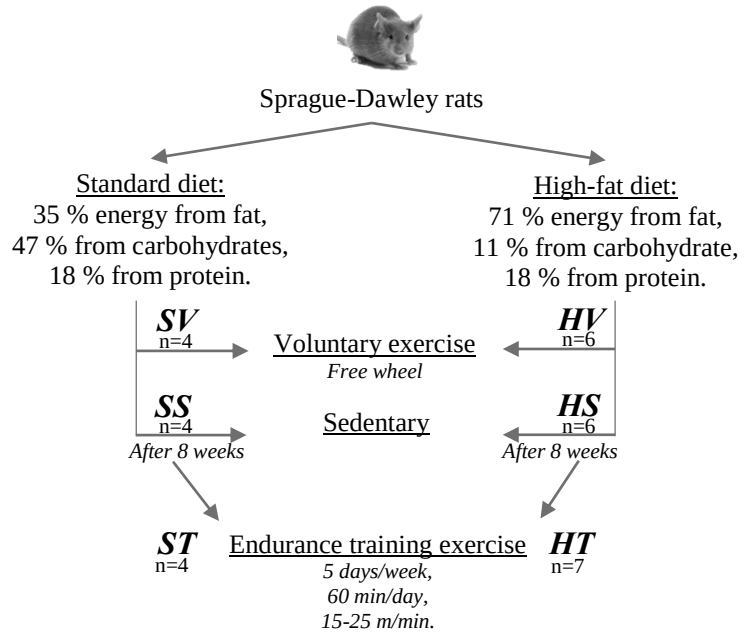


Figure 2.1 – Experimental design according to diet and exercise regimen.

The aqueous phase was transferred to a new tube and RNA was precipitated by adding 0.35-0.5 mL of isopropanol, and left to rest for at least 20 min at -20 °C. After that the samples were centrifuged, and pellets washed with 75 % ethanol (according to the manufactures, in this step, RNA samples can be stored in ethanol for up to 1 year at -20 °C).

In parallel 210-300 μL ethanol was added to remaining phases containing DNA, which were then vortexing gently, after 2-3 min samples were centrifuged. Pellets were washed two times (30min each) with 0.1 M sodium citrate in 10 % ethanol solution. Then DNA pellet was resuspended in 75 % ethanol and 20 min later it was again centrifuged and the pellet dissolved in sterile water. To increase solubility of the samples, they were incubated at 55 °C for 10 min and then centrifuged. The supernatant was recovered and stored at -20 °C.The quantification, of DNA, was performed using NanoDrop ND-1000 spectrometer. Only samples with ratio A260/A280 between 1.7 and 2.0 were considered.

Global DNA methylation analysis

Global DNA methylation was analysed using a commercial kit (MDQ1, Imprint® Methylated DNA Quantification Kit), which is based on antibody recognition of methylated CpG dinucleotyde. For each sample, including positive control provided by the kit (fully methylated Jurkat DNA), 150 ng of genomic DNA were used diluted in the DNA

binding solution provided, and incubated at 37 °C for 60 min, ensuring that the solutions covered the bottom of the wells. The block solution was then added to each well and incubated at 37 °C for 45 min. After, these wells were washed three times with the wash buffer provided and the diluted capture antibody (1:1000) was added to wells and incubated for 60 min. Following this, the wells were washed four times and the diluted detection antibody (1:1000) was added and incubated for 30 min. Again, plate was washed five times and the developing solution was added, protected from light. The colorimetric assay was monitored for colour change to blue for 10 min (maximum time according to manufacturers). Then the stop solution was added (turning reaction yellow). The absorbance was read at 450 nm using Microplate Spectrometer Spectra Max 340. The global DNA methylation levels are presented as percentages relative to SS group mean absorbance, and were calculated using the following equation: $\%\, Global\ Methylation = \frac{(Absorbance\ of\ sample - Absorbance\ of\ Blank)}{(SS\ mean\ absorvance - Absorbance\ of\ Blank)} \times 100$.

cDNA transformation and quantitative Real-Time PCR

The conversion to cDNA was perform on RNA samples stored in 75% ethanol. These samples were centrifuged and the supernatant discarded. RNA samples were then left to dry, in fume hood, overnight. The dryed RNA was dissolved in DEPC water. The quantification was performed using NanoDrop ND-1000 spectrometer, and only samples with ratio A260/A280 between 1.8 and 2.1 was considered. The cDNA synthesis was performed using iScript^{TM} cDNA Synthesis Kit (BIO-RAD). As template was used 1 μg of purified RNA (in two samples was only possible to use 0.25 μg as template) and volumes normalized with nuclease-free water. The complete reaction mix (RNA sample, 1x iScript reaction mix and iScript reverse transcriptase) was incubated for 5 minutes at 25 °C followed by 60 min at 42 °C and for final 5 min at 85 °C. The cDNA was then stored at -20°C.The complete reaction mix was incubated for 5 minutes at 25°C followed by 60 minutes at 42°C and for final 5 minutes at 85°C, in a thermocycler MyCycler from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). The cDNA was then stored at -20°C.

Differences in gene expression were assessed by qRT-PCR. Quantitative gene expression analysis was performed using SYBR Green technology (SsoFast EvaGreen supermix) and CFX96TM Real-Time system from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Reaction solution was obtained with 10 μL of SsoFast EvaGreen supermix, plus 500 nM from each primer, forward and reverse. As template 1 μL of cDNA was used. In each experiment all the samples were measured in duplicates, as well as controls

without template. Samples were amplified using the following conditions: an initial denaturation step of 3 min at 95°C followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. As reference gene *β-actin* was used, and the genes tested were *SREBP-2*, *ABCA1*, *ABCG5*, *ABCG8* and *NPC1L1*. To reduce misleading results, signals above 38 Cq were not considered. The fold induction was determined using the ΔΔCq method.

RNA formaldehyde agarose electrophoresis

In order to analyse RNA quality, RNA extracted from colon samples were separated in a formaldehyde agarose gel which allows the identification of bands correspondent to 28S, 18S and 5.8S ribosomal RNA, well defined in high quality RNA. For comparison was also analysed RNA extracted from culture cells using TRI reagent protocol, as described, and RNA extracted from culture cells using a commercial kit (protocol described in Chapter 3). Briefly, 1 μg of RNA sample was mixed with loading buffer (0.06 % (v/v) formamide, 0.02 % (v/v) formaldehyde, 0.8 mM EDTA, 4 % glycerol and 0.04 % bromophenol blue). Then samples were incubated for 3 min at 65 °C and immediately following chilled on ice. Samples were load in 1.2 % (m/v) agarose in MOPS gel buffer (20 mM MOPS, 5 mM sodium acetate, 0.5 mM EDTA, pH 7.0, supplemented with 1.8 mL of formaldehyde and 5 μL of Safe-Green™). Gel run at 50 V during 40 min in running buffer (20 mM MOPS, 5 mM sodium acetate, 0.5 mM EDTA, 2 % formaldehyde). Gel was then visualized using UV transillumination detection system Chemi Doc XRS (Bio-Rad).

Protein extraction and western blot analysis

With the aim of analysing differences in protein levels in the colon samples, 100-150 mg of tissue, was homogenised with 10 x (m/v) mL of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 0.1 % SDS, 1 % Sodium deoxycholate, freshly supplemented with 20 mM sodium orthovanadate (Na_3VO_4) and sodium fluoride (NaF), fosfatase inhibitors, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor, and 40 μg/mL protease inhibitory cocktail (Roche, Mannheim, Germany)). In some assays, homogenization was accomplished using a polytron tissue homogeneizer, conveniently maintaining the samples at 4 °C, for method comparison. Samples were incubated with lysis buffer during 10 min, and periodically vortexing. After centrifugation at 10000 g, 4 °C during 10 min supernatant – total protein extract – was collected to a new eppendorf tube. The same process of extraction was performed using a different lysis buffer, for comparison, the RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 %

NP-40, 2 mM EDTA, freshly supplemented with 20 mM Na_3VO_4 and NaF, fosfatase inhibitors, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor, and 40 µg/mL protease inhibitory cocktail). The protein concentration of the samples was determined though DC protein assay kit from Bio-Rad according their instructions. Blank was defined with respective lysis buffer, and calibration curve was performed with BSA. The storage of protein samples was at -80 °C, until further analysis.

Proteins were analysed by western blot, a widely used technique which allows the separation and identification of specific proteins, by the use of specific antibodies (Mahmood and Yang, 2012). 20 to 40 µg of protein were separated by molecular weight on 10 % SDS- Polyacrylamide gel electrophoresis and transferred onto Hybond-P polyvinylidene difluoride membranes (Westran®, 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), for at least 2 hours under gentle agitation at room temperature and then washed three times with TPBS before incubation overnight at 4 °C with β-actin primary antibody. After three washes with TPBS, membranes were incubated, for 1 hour under agitation, with anti-mouse antibody (in 5 % (w/v) non-fat dry milk in TPBS), which recognizes the primary antibody. For immunoreactive bands detection 2 min incubation with Clarity™ Western ECL Substrate mixture were made and bands visualized by the chemiluminescence detection system Chemi Doc XRS (Bio-Rad). Membranes were stained by overnight incubation with 0.5 % (v/v) Chinese Ink, in 0.3 % (v/v) Tween 20 in PBS, pH 7.4.

Statistical analysis

All the results are presented as means ± SEM. For statistical analysis GraphPad Prism 5.0 software (San Diego, CA, USA) was used, and Two-way ANOVA with Bonferroni post-test for multiple group comparison. Differences were considered significant when p value was $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

2.3 - Results

DNA methylation can be modified by lifestyle, and be a epigenetic marker of the cross talk between lifestyle and diseases (Zhang et al., 2014). We evaluate the global DNA methylation levels in the colon samples. *Figure 2.2 A* represents the effect on global DNA methylation of the high-fat diet consumption with or without voluntary physical activity. In *Figure 2.2 B* is represented the effect of endurance training introduced half the

way into the experiment. The increase of fat in daily diet increased global DNA methylation, relative to standard diet fed rats. In case of the standard diet there were no statistically significant differences between exercise regimens. On the contrary, voluntary physical activity seems to counteract the increased global DNA methylation caused by intake of high-fat which suggest an interaction between diet type and voluntary physical activity. Indicating that global DNA methylation is influenced by both diet and exercise regimen.

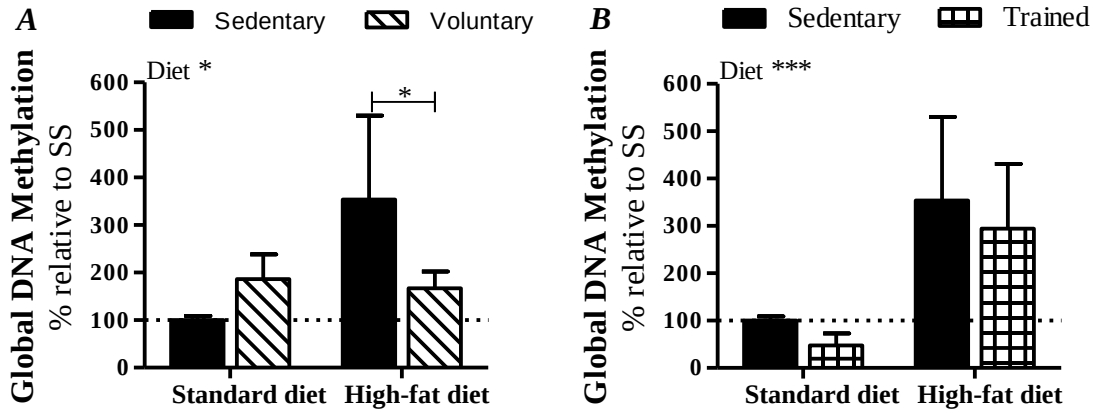


*Figure 2.2 – Global DNA methylation, in percentage of control, in colon samples. A – Effect of voluntary physical activity. B – Effect of endurance training. Results are presented as % relative to SS group. Values are mean ± SEM of four independent experiences. For statistical analysis Two-way ANOVA followed by Bonferroni post-hoc-test for comparison of multiple groups were performed, (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).*

As internal control of the method was used a fully methylated control – human methylated Jurkat DNA, which is 80-85 % methylated (analysed by Liquid Chromatography - Mass Spectrometry). This internal control presented absorbance values on average equal to the SS group, however values oscillated significantly between trials, as did samples. Thus our results may only reflect tendencies in global DNA methylation values.

In order to understand if high-fat diet or physical activity influenced cholesterol metabolism and transport in colon, we evaluated gene expression changes through qRT-PCR technology, in these tissue samples. Effects on gene expression of *SREBP-2*, the main regulator of cholesterol synthesis, *ABCA1* determinant for HDL levels; *ABCG5* involved in cholesterol excretion, and *NPC1L1* a transporter related to cholesterol absorption, were investigated. Our results show that in colon tissue there not statistically significant differences were found in *SREBP-2* gene expression induced by diet and voluntary physical activity, (*Figure 2.3 A*). Similarly *ABCA1* (*Figure 2.3 B*), neither was induced by diet nor exercise. On the other hand, *ABCG5* expression (*Figure 2.3 C*) seems to be affected, only when rats were fed with a high-fat diet, inducing its expression. In that

case, voluntary physical activity seems to reduce *ABCG5* expression, counteracting the effect of high-fat consumption and bringing values close to those of standard diet. Showing an interaction between diet and physical activity at this level. Relatively to *NPC1L1* (*Figure 2.3 D*), there was no effect due to diet. However, exercise had a markedly effect on *NPC1L1* expression, voluntary physical activity decreased its expression in both standard diet and in high-fat diet.

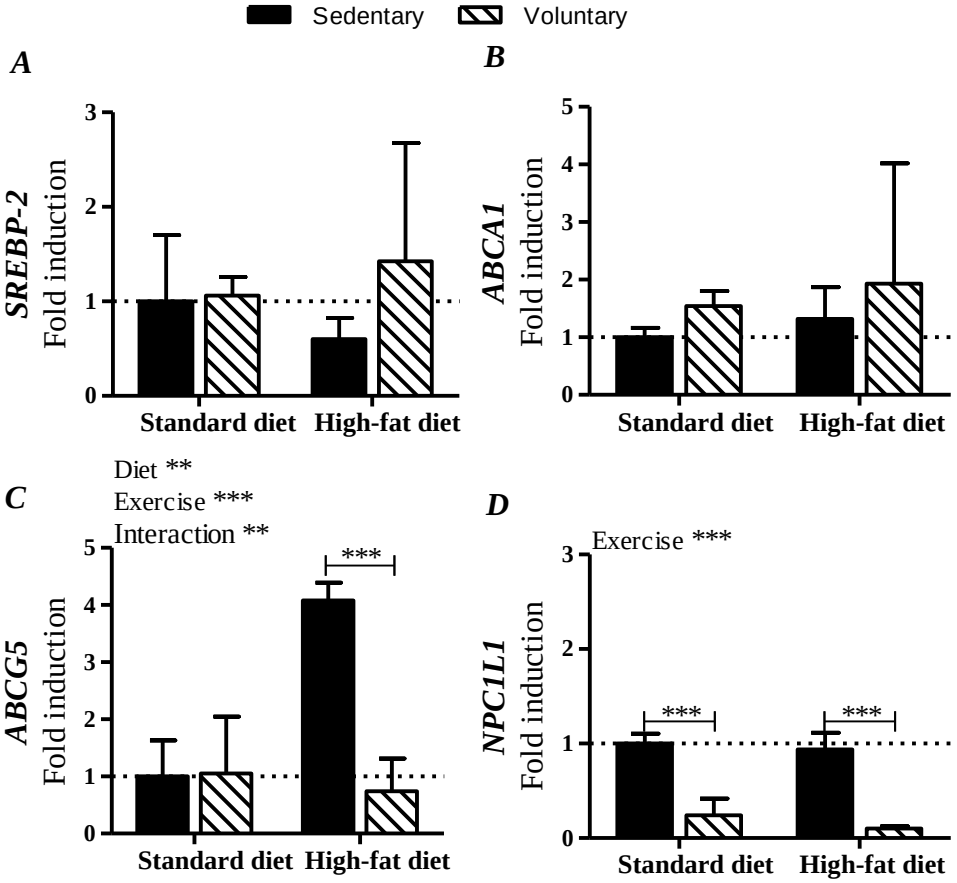


*Figure 2.3 – Analysis of SREBP-2 (A), ABCA1 (B), ABCG5 (C) and NPC1L1 (D) gene expression using qRT-PCR technology, relatively to sedentary vs voluntary physical activity, in both diets. The results are presented as fold induction relative to SS group, using β-actin as endogenous control. Values are mean ± SEM of four independent experiences. For statistical analysis Two-way ANOVA followed by the Bonferroni post-hoc-test for comparison of multiple groups were performed, (*p < 0.05, **p < 0.01, ***p < 0.001).*

In this analysis, the effect of endurance training as a therapeutic measure to revert the deleterious effects of high-fat diet and obesity was also evaluated. Once again, neither *SREBP-2* nor *ABCA1* gene expression were affected by this exercise regimen for 8 weeks, in both diets (*Figure 2.4 A, B*). The expression of *ABCG5* (*Figure 2.4* C) higher in high-fat diet, was not changed, by endurance training. However, there was high variability in the results obtained in HT group. For *NPC1L1* (*Figure 2.4 D*) gene expression, the effect of endurance training seems to be dependent of calories source in diet. Because under the standard diet, endurance training reduces the expression of *NPC1L1*. Whereas when rats were fed with high amount of fat, endurance training did not change *NPC1L1* expression, relatively to sedentary lifestyle.

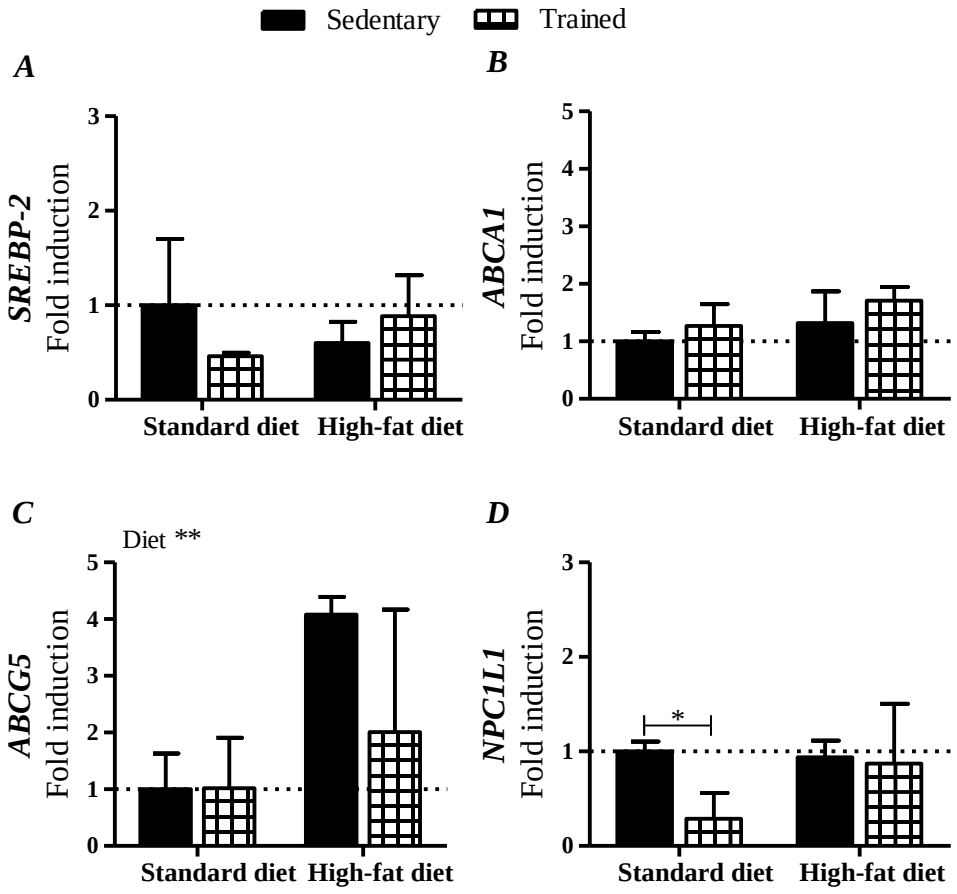


*Figure 2.4 – Analysis of SREBP-2 (A), ABCA1 (B), ABCG5 (C) and NPC1L1 (D) gene expression using qRT-PCR technology, relatively to sedentary lifestyle vs adhesion to endurance training, in both diets. The results are presented as fold induction relative to SS group, using β-actin as endogenous control. Values are mean ± SEM of four independent experiences. For statistical analysis Two-way ANOVA followed by the Bonferroni post-hoc-test for comparison of multiple groups were performed, (*p < 0.05, **p < 0.01).*

Even though there were differences in gene expression, and, in fact we used only RNA samples with ratios between 1.8 and 2.1 as recommended. There was high variability in our results within the same group. In order to have more confidence in our results we analyse our RNA extracts in denaturing conditions using formaldehyde agarose gels. In our analysis, we included a RNA sample extracted from HepG2 cells using a SV Total RNA Isolation System from Promega (Madison, WI, USA), and one extracted with TRI reagent with the same protocol used in tissue samples. As presented in *Figure 2.5*, not all the colon tissue RNA samples maintained integrity, as evidenced by the reduced ratio between 28S:18S rRNA band intensity, and presence of smeared bands (Aranda et al., 2013). In fact, the extraction protocol using TRI reagent seems itself to effect integrity when compared with the commercial kit. However, since our original tissue samples were stored in TRI reagent and we aimed at the extraction of both DNA and RNA from the same sample, TRI reagent was our best choice. A study published in 2010 showed that TRIzol preserves RNA quality well in long term storage (Ma et al., 2010). Although since colon tissue was preserved intact it is possible that TRI reagent in which samples were kept did not preserve evenly the tissue leading to unwanted degradation.

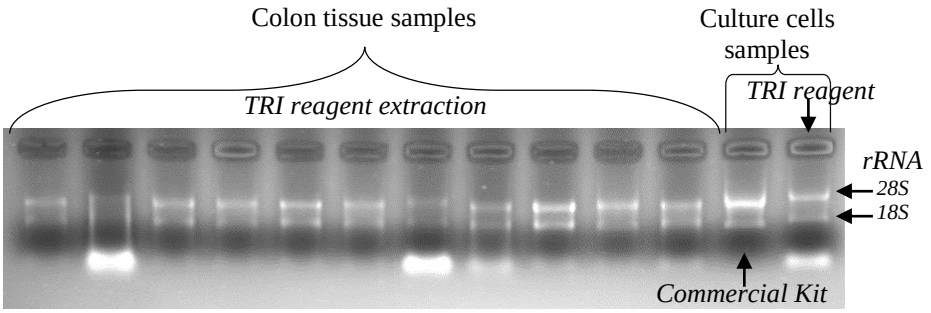


Figure 2.5 – Analysis of RNA quality extracted from colon samples in a denaturing 1.2 % agarose gel using formaldehyde. RNA extracted from culture cells with TRI reagent and commercial kit were included for comparison. Gel stained with Safe-Green™ and visualized under UV light using Chemi Doc XRS detection system.

One aim of this work was analyse changes in specific protein levels in the different conditions of the study. Namely, study the activation of the transcription factor SREBP-2, which occurs by a proteolytic process and therefore should be assessed by western blot technique. We performed protein extraction of colon tissue, using two different lysis buffer, with and without the use of homogenizer. In all cases we obtain a high protein concentration, however, we were unable to obtain an intact protein extract. Upon performing a western blot and staining our membrane using Chinese ink, we realized that

none of the protein extracts presented clear-cut bands over all molecular weights, instead we observed a dragging along the membrane which suggest protein degradation (data not shown). In fact none of the antibodies we tested worked, even β-actin, normally used as loading control, making our study unfeasible.

2.4 - Discussion

The epigenetic mechanisms are highly influenced by environmental and lifestyle factors, including diet and exercise, which lead to adaptations in epigenome, with impact on gene expression (Huidobro et al., 2013). Regular physical activity, is a component of lifestyle with known benefits on health, and an adaptation of epigenetic marks have been described in response to physical activity, with different effects according to the tissue (Ling and Rönn, 2014). In an intervention study in sedentary health man, the inclusion of 1 h of aerobic exercise twice a week, during 6 months, resulted in variations in DNA methylation in gene promoter regions. In skeletal muscle the main variations were a decrease in global methylation, while in adipose tissue, the majority of genes in which changes in methylation state were found, they were due an increase in methylation (Nitert et al., 2012; Rönn et al., 2013). Another study evaluated global DNA methylation in leukocytes of older individuals (around 70 years old), and showed that physical activity seems to reduce global DNA methylation in healthy individuals. However, it is important to keep in mind that levels of methylation in leukocytes vary with age, and increase methylation levels could be an indicator of inflammation (Luttropp et al., 2013). These results show that epigenetic markers, besides being affected by exercise, are also time dependent.

High-fat diet consumption on alterations of DNA methylation, has been described to have a higher influence in prenatal and early postnatal state. An increase on DNA methylation state could be related to intake of phosphatidylcholine, found in fat from animal sources, which increase methyl-donor availability (Waterland and Rached, 2006). Therefore, the influence of diet on DNA methylation has been, mainly, attributed to levels of methyl donors on diet, such as methionine, choline and folate, which are fundamental for DNMTs activity (Varela-Rey et al., 2013). Early development stages are probably more susceptible to epigenetic alterations (Huidobro et al., 2013).

In the present study, the major source of variation in global methylation levels seem to be the diet, rather than exercise. Rats fed with a high-fat diet presented higher

levels of global DNA methylation. Even so, voluntary physical exercise seems to normalise values of global methylation levels in animals fed with both diets. While the later introduction of endurance training did not seem to change significantly the levels of DNA methylation. Rats were only subject to endurance exercise 8 weeks after the beginning of high-fat diet consumption, after the metabolic deleterious effects of the high-fat diet had ensured. It seems that the 7 weeks of exercise were not able to reverse the effects of high-fat diet. Since both diets contained the same amount of folic acid and methionine, as well as choline, (indicated in *Suplementary Table S.1*) and all the groups of treatment ingested the same amounts of food without statistics differences (Gonçalves et al., 2014b), the effect of increased DNA methylation in response to high-fat diet could not be explained by different consumption of these nutrients.

DNA hypomethylation is usually associated to demethylation of repetitive sequences which may lead to genomic instability and induced aberrant activation of certain genes as oncogenes (Ling and Groop, 2009). Usually it is associated with aging and also with carcinogenesis, such as colorectal cancer (Huidobro et al., 2013). In cancers, however, hypermethylation of specific genes, is also observed and is responsible for silencing of critical genes, such as DNA repair genes *MLH1* and *MGMT* (Hinrichsen et al., 2014; Li et al., 2013b). Gene promoter's hypermethylation is associated with gene silencing, specifically genes which prevents cancer development. In fact, colon cancer is the most associated with high frequency of CpG island hypermethylation (Ehrlich, 2002). Our results may link high-fat diet to this increase in DNA hypermethylation. However, it is fundamental further studies in order to analyse promoters' specific methylation, using for example methylation-specific PCR. It is important to notice that changes in global methylation are not direct indicators of which processes are being affected. But the variations in the epigenome may be one of the justification for differences in gene expression.

In order to understand the effects of different source of calories in diet (carbohydrates vs fat), and the effect of different regimens of physical activity we analysed the expression of transporters related to cholesterol absorption and secretion, as well as the main regulators of cholesterol synthesis, in colon samples. Our results show that gene expression of *SREBP-2* seems not be dependent of diet or exercise, in colon, although more than its expression, its activation is relevant in the stimulation of cholesterol synthesis. However we were not able to analyse protein levels in these samples by western blot as mentioned previously.

The ABCA1 transporter, is fundamental in regulation of cellular cholesterol export, where mediates the transport of phospholipids and cholesterol into HDL particles. This transporter is ubiquitously expressed, however, tissue-specific *ABCA1* knockout, showed that the tissues in which *ABCA1* is more determinant for HDL circulating levels are the intestine and the liver (Ikonen, 2008). Previous work found higher HDL levels due to diet manipulation, independently of the exercise regimen [presented in Supplementary information: *Table S.2* (Gonçalves et al., 2014a)], however in our analysis we did not find significant effects on *ABCA1* gene expression of diets or exercise. These results suggest that if the effect observed on HDL levels is dependent of *ABCA1* transporter expression those changes should be mainly due to effects in the liver.

The blood biochemical analysis performed at the end of the all period of diet manipulation and physical activity intervention, published in (Gonçalves et al., 2014a), showed that the plasma total cholesterol levels are dependent on both diet and exercise regimen (Supplementary information: *Table S.2).* In animals fed with standard diet, cholesterol levels were reduced by both voluntary physical activity and, particularly, by endurance training. On the other hand, the cholesterol levels of high-fat fed rats were higher than standard diet fed rats. In this case, voluntary physical activity result in higher cholesterol levels, while endurance training maintain its capacity of reduce cholesterol levels relatively to sedentary lifestyle.

Since none of the diets administered constitutes a source of cholesterol, the levels of cholesterol in these rats corresponds solely to endogenous synthesis and the mechanisms of excretion and re-absorption. The transporters ABCG5/ABCG8 promote efflux of cholesterol, at intestinal level, while also promoting biliary sterol secretion (Yu et al., 2002). Over all, their increased expression is expected to be correlated with a decrease in cholesterol levels. Since the major route for cholesterol excretion from body is its elimination as biliary cholesterol which is not re-absorbed (Li et al., 2013a; Meissner et al., 2010). Changes in these transporters may explain how exercise and diet affect cholesterol metabolism. *ABCG5/G8* are only effective when both are concomitantly expressed, because if only one of them is expressed serves as a non-functional half-transporter (Yu et al., 2002). In our work, we were not able to analyse *ABCG8* expression, because in almost all the samples Cq values were above the 38 cut-off, making the result unreliable. In order to try estimate the effect of diet and exercise on sterol excretion we considered the effect on *ABCG5*, as representative of the changes on functional dimer transporter. However, is

important to notice that even the analysis of both *ABCG5* and *ABCG8* gene expression, does not give information about if the functional dimer is formed.

The effect on *ABCG5* gene expression seems to be dependent both of the diet and the exercise regimen. The results show that high-fat fed rats have a higher expression of *ABCG5* relatively to standard diet fed rats. Which may reflect a higher biliary cholesterol dependence to fat absorption, and explain the higher levels of plasma cholesterol in high-fat fed rats as reported (Gonçalves et al., 2014a). In addition, the overexpression of these transporters (*ABCG5*/*ABCG8*) has been found to occur in response to high levels of dietary cholesterol and stimulation of LXRα (*ABCG5*/*ABCG8* gene expression is LXRα dependent) (Yu et al., 2002). LXR is also stimulated by phytosterols and derivate, namely dietary β-sitosterol, stigmasterol and campesterol (Kim et al., 2008). These phytosterols are present in higher concentrations in corn oil (rich in polyunsaturated FA), relatively to olive oil (rich in monounsaturated FA, low in phytosterols) (Howell et al., 1998). Since high fat diet contains more corn oil than standard diet (Supplementary information *Table S.1*), this may explain the higher expression of *ABCG5* observed in high-fat diet fed rats. Diets with high consumption of phytosterols has been associated with lower levels of plasma cholesterol, once that phytosterols may decrease incorporation of dietary cholesterol in mixed micelles reducing its absorption (Yu et al., 2014). However, *ABCG5* expression was also influenced by exercise regimen. Practice of voluntary physical activity counteracted the effect of dietary fat consumption, reducing neutral sterols loss, which may explain why HV rats presents higher plasma cholesterol levels. On the other hand, the incorporation of endurance training after establishment of metabolic diseases, did not significantly reduce *ABCG5* expression, this may explain why HT rats have lower plasma cholesterol levels relatively to HV. The homeostasis of cholesterol levels depends not only of how much is secreted but also how much cholesterol is absorbed.

NPC1L1 is expressed in brush border membrane of enterocytes and is responsible for the absorption of free cholesterol. The excretion of biliary cholesterol could be counteracted by intestinal cholesterol absorption dependent on *NPC1L1* expression (Betters and Yu, 2010). Our results show that *NPC1L1* expression is not affected by diet. The effect of voluntary physical activity, is the same in both diets, reducing *NPC1L1* gene expression, which is expected to result in lower re-uptake of cholesterol. In these animals, biliary cholesterol is the only source of cholesterol in the intestine lumen, because they were fed in diets without animal fat in their composition. A similar reduction in cholesterol absorption (reduced *NPC1L1*) was also observed in rats with higher consumption of

cholesterol with voluntary running in wheel (Meissner et al., 2010). On the other hand, the implementation of an endurance training only results in reduction of *NPC1L1* in standard diet fed rats, suggesting an interaction between diet and exercise effect. Taken together, these results seem to show that, in high-fat diet, when secretion of biliary cholesterol is higher (increased *ABCG5*) the reabsorption of cholesterol is also enhanced, suggesting a requirement of cholesterol. Once that this result is only observed in high-fat fed rats this could result from the requirement of cholesterol to bile acids production and therefore excretion to emulsification of fats in order to their absorption (Yu et al., 2002). It is possible that in high-fat fed rats endurance training did not reduce *NPC1L1* expression, because of the increased energy requirements caused by heavy exercise and consequent increased need of cholesterol for bile acids production and increased the absorption of fats, the major source of energy available.

Chapter 3 - Experimental Part B:
Effects of Naturally Occurring Dietary Flavones on Liver Cholesterol Metabolism, in vitro

3.1 - Chapter Introduction

Lipid metabolism has a strong influence in diseases, such as CVD, responsible for about one-third of all global deaths and the most important cause of morbidity (Afzali et al., 2013; WHO/FAO, 2003), T2DM and NAFLD, which affect approximately 30 % in western populations (Kim and Younossi, 2008). In fact, dyslipidemia is one of the parameters that define MetS, and increase risk for the development of these disorders (Kaur, 2014). In our daily life prevention of these diseases may be achieved through changes in lifestyle, namely physical activity and diet (WHO/FAO, 2003).

Flavones belong to the flavonoid family of phytochemicals with health improving properties (Kumar and Pandey, 2013). Luteolin, and its most abundant glycoside form Luteolin-7-O-Glucoside, are naturally occurring flavones present in aromatic plants (such as oregano, parsley, thyme, perilla, chervil, anise and sage) but also in other plants and vegetables (such as olives, carrots, artichokes cacao, celery, peppers, spinach, broccoli, cauliflower, pumpkin and lemon) (Amin et al., 2009; Bhagwat et al., 2014; López-Lázaro, 2009). These widely occurring compounds have been associated anti-inflammatory, antioxidant, antimicrobial and anti-cancer activities. In addition to being cardio-protective and antidiabetic (Ding et al., 2010; Liu et al., 2014, 2011; López-Lázaro, 2009).

In a previous work performed in our laboratory, L7G was provided in the diet of healthy rats during 7 consecutive days and resulted in a decrease in plasma LDL and total plasma cholesterol levels (Azevedo et al., 2010). These effects could be attributed to the observed reduction of HMGCR gene expression and potential decrease in cholesterol synthesis, combined with the induction of PPARα and its target CPT1 gene expression, which could favour FA β-oxidation and consequently reduce fat accumulation (Sá et al., 2015). These results indicate a statin-like and fibrate-like action mechanisms of L7G and highlight the potential of this compound in the prevention of dyslipidaemias and metabolic diseases. However, although L7G is the most abundant form of this flavone in aromatic plants, fruits and vegetables, and most easily provided by our diet, it is generally accepted that it is the aglycone compound – L – that is absorbed and not L7G (Park and Song, 2013). The hydrolysis of the glycosides by β-glucosidase enzyme in the gut has been described to occur before intestinal absorption. However, there are evidences that glycosylated forms could be absorbed without modifications. This would result in liver exposure to the glycosylated form, as well as the agycone, rising questions as to whether

L7G could also be biologically active (Park and Song, 2013). In this context the present study focuses on understanding the effects of both forms of this flavone: the aglycone L, *Figure 3.1 A* and glycoside L7G, *Figure 3.1 B*, on lipid metabolism through an *in vitro* approach. Human hepatocellular carcinoma HepG2 cells were used to evaluate L and L7G effects on lipid metabolism, in order to compare if both forms produces the same outcomes, and if the effects *in vitro* in human liver cells are congruent with the previous evidences. The effect of these flavones were compared to those of Simvastatin (SIMV) (HMGCR inhibitor), *Figure 3.1 C*, a statin used as pharmacological drug in treatment of dyslipidaemias, and also Fenofibrate (FF), *Figure 3.1 D*, a pharmacological drug that function as a PPARα agonist.

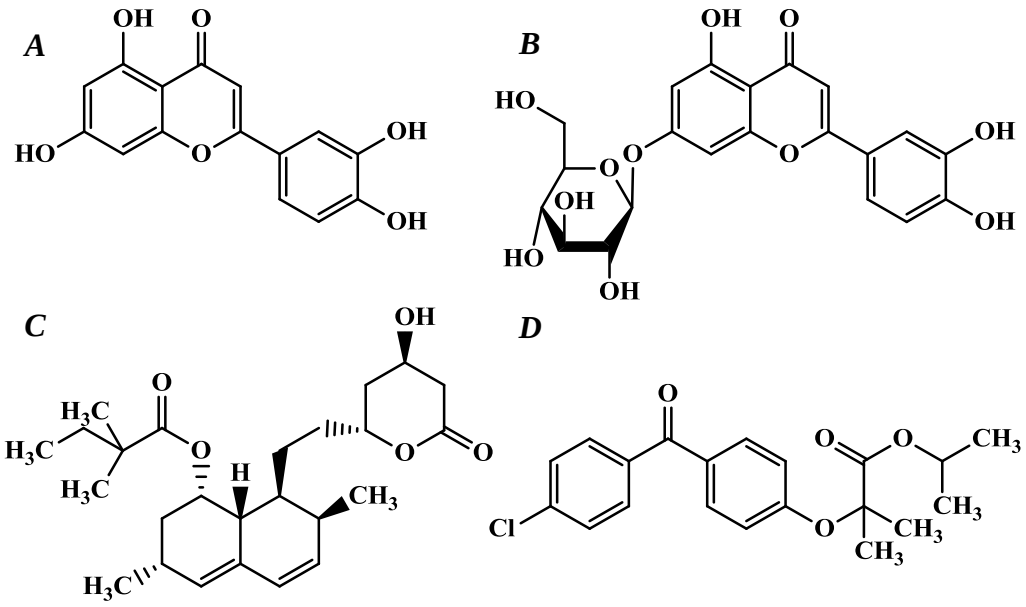


Figure 3.1 – Chemical structure of Luteolin (A), Luteolin-7-O-Glucoside (B), Simvastatin (C) and Fenofibrate (D).

3.2 - Material and Methods

Reagents, antibodies and primers

Both Luteolin and Luteolin-7-O-Glucoside were purchased to Extrasynthese (Genay, France). Fenofibrate was acquired to Tocris Bioscience (Bristol, UK). While Simvastatin and 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were acquired to Sigma-Aldrich (St. Louis, MO, USA). Minimum essential medium Eagle (MEM), antibiotic-antimycotic solution, sodium pyruvate, HEPES, sodium bicarbonate and Trypsin-Ethylenediaminetetraacetic acid (Trypsin-EDTA) solution used in cell culture were purchased from Sigma-Aldrich (St. Louis, MO, USA), as well as bovine

serum albumin (BSA), Triton X-100 and Tween-20 which were acquired to the same company. While fetal bovine serum (FBS) was acquired to Biochrom KG (Berlin, Germany). Sodium deoxycholate used in lysis buffer was purchased to Fluka/BioChemika (Buchs, Switzerland). The kit used in quantitative determination of cholesterol, Cholesterol-LQ CHOD-POD Liquid was requested to Spinreact (Girona, Spain). Chloroform used in cholesterol extraction was acquired from José Vaz Pereira, Lda. (Sintra, Portugal).

The DC protein assay kit to determination of protein concentration and Clarity™ Western ECL Substrate to chemiluminescent western blot detection, as well as iScriptTM cDNA Synthesis Kit and SsoFast EvaGreen supermix, utilized in quantitative real-time PCR were acquired from Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The kit used in RNA extraction SV Total RNA Isolation System was purchased from Promega (Madison, WI, USA). All others reagents were of analytical grade.

The antibody against SREBP-2 (ab30682) was purchased to Abcam (Cambridge, UK). Anti-PPARα antibody (sc-9000) and anti-mouse secondary antibody containing IgG horseradish peroxidase were acquired to Santa Cruz Biotechnology (Santa Cruz, CA, USA). Both antibodies against total ACC (C83B10) and p-ACC (D7D11), as well as anti-rabbit secondary antibody containing IgG horseradish peroxidase were acquired to Cell Signalling Technology (Danvers, MA, USA). The antibody against β-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA). The primers used in qRT-PCR are indicated in *Table 3.1*. Primers sequences were either found in literature or designed by ourselves, and its specificity confirmed using Primer-Blast tool provided by NCBI. Selected primers were then manufactured by STAB VIDA, Lda, (Caparica, Portugal).

Table 3.1 – Primers Sequences used in qRT-PCR.

Gene	***Primers Sequence***	***NCBI Reference Sequence:***	***Ref.***
HMGCR	F: CTGGGGAATTGTCACTTATGG R: TCTTGTAAATTGATCTTCGACCTG	NM_000859.2	(Ginanni Corradini et al., 2013)
LDL receptor	F: CAACTTTGACAACCCCGTCT R: GGCAATGCTTTGGTCTTCTC	NM_000527.4	Designed
ABCA1	F: ATCTCATAGTATGGAAGAATGTGAAGCT R: GTACAACTATTGTATAACCATCTCCAAA	NM_005502.3	(Racine et al., 2010)
CPT1	F: AGGGATGCAAATCTTGTTGAGT R: TTTGGTTAGTGCATTCCAGATG	NM_001876.3	Designed
β-actin	F: GAGCGGGAAATCGTGCGTGAC R: GCCTAGAAGCATTTGCGGTGGAC	NM_001101.3	(Peschel et al., 2007)

Culture conditions of HepG2 cell line

HepG2 cell line derived from human hepatocellular carcinoma was purchased to American Type Culture Collection. The cells were maintained in monolayer culture in 75 cm^2 TPP polystyrene tissue culture flasks at 37 °C in an atmosphere of 5 % CO_2, using MEM supplemented with 10 % inactivated FBS, 1 % antibiotic-antimycotic solution, 1 mM sodium pyruvate, 10 mM HEPES and 2.2 g/L sodium bicarbonate. Medium was replaced twice per week and culture was maintained with weekly passages at the time they reached approximately 90 % confluence. To that, cells were detached using Trypsin-EDTA solution, neutralized using fresh medium, and resuspended. Their cellular density were determined using Neubauer chamber. The cellular density is characteristic of each test and is described in the following sections. In each test, control conditions were always cells incubated with 0.5 % DMSO in the same incubation conditions as test compounds.

MTT reduction assay

The MTT reduction assay relies on the metabolic activity of cells to assess their viability once that only viable cells have the ability to reduce, by the action of mitochondrial reductase enzyme, the yellow compound MTT to (2Z, 4E) -5- (4,5-dimethylthiazol-2-yl) -1,3-difenilformazan, forming violet crystals of MTT-formazan. As a colorimetric assay it is a useful tool, being practical and easy to perform (Fotakis and Timbrell, 2006). The cells in the suspension obtained on the occasion of cells passage (as described) were plated, in 24-wells plates, at $2.5x10^5$ cells/mL cell density and allowed to conveniently adhere to the well, for 48 h. The medium was then discarded and replaced with fresh medium containing test compounds. Here, cells were incubated with L, L7G and FF at the final concentration of 10, 20 and 30 μM, while SIMV was incubated with 5, 10 and 20 μM for 48 h. In the last hour of the 48 h period, MTT was added to the wells to achieve the concentration of 5 mg/mL, maintaining cells at 37 °C in an atmosphere of 5 % CO_2. At the end of incubation time, medium was discarded and MTT-formazan crystals, formed by metabolically viable cells, were dissolved with DMSO/Ethanol (1:1) solution. After complete crystals dissolution, 200 μL were collected into a 96-well plate and absorbance was measured using Microplate Spectrometer Spectra Max 340, at 570 nm and 690 nm. The percentage of viable cells was estimated using the following formula: $\% \, Cell \, Viability = \frac{Abs_{570} - Abs_{690} \, (sample)}{Abs_{570} - Abs_{690} \, (control)} \times 100$ and results expressed as percentage relative to the control (cells incubated with 0.5 % DMSO).

Total cellular cholesterol quantification

Cellular cholesterol was quantified in cells plated in 6-wells plates at $2.5x10^5$ cells/mL using 2 wells per condition. Cells were incubated with compounds as described above. Here, cells were incubated with L and L7G at 20 μM, and SIMV at 10 μM during 24 h. Or with L, L7G and SIMV alone or in co-incubation, at the same concentrations, for 72 h. At the end of the incubation period, medium was collected and cells detached trough incubation with Trypsin-EDTA solution. The medium recovered was used to inactivate trypsin, and then everything was collected to a tube, and henceforward kept on ice. Wells were washed with fresh phosphate-buffered saline (PBS) to ensure that all the cells were gathered, and tubes centrifuged at 500 g, 4 °C for 5 min.

The pelleted cells were resuspended in 500 μL of supernatant and transferred to eppendorfs tube, and centrifuged again. In this step, all the supernatant was discarded and 300 μL of lysis buffer (50 mM Tris-base, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 0.1 % SDS, 1 % Sodium deoxycholate, freshly supplemented with 20 mM Na_3VO_4 and NaF, fosfatase inhibitors, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor, and 40 μg/mL protease inhibitory cocktail (Roche, Mannheim, Germany)) was added. Cells were maintained on ice with lysis buffer for 10 min, with periodic vortexing. At the end of this time a 20 μL sample was taken for protein quantification, as described below. Subsequently, in the fume hood, 200 μL of chloroform were added to the samples followed by gentle stirring. When two defined phases formed, the bottom one (more dense – organic phase) was collected to another tube, repeating the process three times in a row with the lighter phase. Cholesterol is soluble in chloroform, therefore the addition of this organic solvent allows the separation of cholesterol from the remaining cellular components. Tubes containing the combined organic phases were left to dry, overnight in the fume hood.

The quantification of cholesterol was achieved using the commercial kit Cholesterol-LQ CHOD-POD Liquid from Spinreact. To that dried samples were dissolved in 80 μL ethanol and submitted to ultrasound during 5 min, followed by centrifugation at maximum speed of bench centrifuge for 2 min. In a 96-wells, 25 μL of each sample was mixed with 250 μL reagent R supplied. Equally 25 μL of ethanol was used to define plate blank, and 2.5 μL of aqueous cholesterol standard at concentration of 2000 μg/mL. After addition of reagent R, the plate was incubated for 5 min at 37 °C, and optical density measured at 505 nm using Microplate Spectrometer Spectra Max 340. The cholesterol present in the sample originated a coloured complex - quinonimine, once samples cholesterol react

with reagent R (containing Cholesterol esterase, Cholesterol oxidase and Peroxidase enzymes).According to manufacturer resulting colour is stable for 60 min, and directly proportional to cholesterol concentration. The cellular cholesterol was estimated with the following formula: $\mu g\ cholesterol = \frac{Abs_{505}\ (sample)}{Abs_{505}\ (control)} \times 2000\ \mu g/mL \times 80\ (\mu L\ solution)$. The results were then normalized with the mg of protein of respective samples, and are presented as percentages relative to control. This protocol was developed by adaptation of the process described in (Chu et al., 2010).

Protein extraction and western blot analysis

With the aim of analysing differences in protein levels HepG2 cells were plated in 6-wells plates at $2.5x10^5$ cell/mL. After 48 h for complete adherence, cells were incubated with L and L7G at 20 μM, and SIMV at 10 μM incubated alone or in co-incubation, during 16 h. Ending the incubation period, medium was discarded and cells washed with PBS. Washed cells were then scraped in cold PBS and collected to eppendorf tube, ensuring that all the cells were gathered, and henceforward kept on ice. After of a centrifugation at 500 g, 4 °C for 5 min, supernatant was discarded and was added 80-100 μL of lysis buffer. Cells were maintained in ice with lysis buffer during 10 min, and periodically vortexing. Followed by centrifugation at 10000 g, 4 °C during 10 min in which supernatant – total protein extract – was collected to a new Eppendorf tube. The protein concentration of the samples was determined though DC protein assay kit from Bio-Rad according their instructions. Blank was defined with lysis buffer and calibration curve was performed with BSA. The storage of protein samples was at -80 °C, until further analysis.

Proteins were analysed by western blot, a widely used tecnique which allows the separation and identification of specific proteins (Mahmood and Yang, 2012). To perform that analysis 20 to 30 μg of protein were separated by molecular weight on 10 % SDS-Polyacrylamide gel electrophoresis and transferred onto Hybond-P polyvinylidene difluoride membranes (Westran®, 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), for at least 2 hours under gentle agitation at room temperature and then washed three times with TPBS before incubation overnight at 4 °C with primary antibody. After three washes with TPBS, membranes were incubated, for 1 hour in agitation, with secondary antibody (in 5 % (w/v) non-fat dry milk in TPBS). . For immunoreactive bands detection 2 min incubation with Clarity™ Western ECL Substrate mixture were made and bands visualized by the chemiluminescence detection system Chemi Doc XRS (Bio-Rad). Band

area intensity was quantified using Quantity One software from Bio-Rad. The proteins analysed were SREBP-2, SREBP-1, PPARα, ACC, p-ACC and finally β-actin which was used as loading control. Control conditions were considered to correspond to 1, arbitrary units.

RNA extraction

With the purpose of performing a qRT-PCR in cell samples, total RNA extraction was executed using SV total RNA extraction system (Promega), following the producer's protocol. To that, HepG2 cells were plated in tissues culture dishes TPP (Ø 60 × 16 mm) at $2.5x10^5$ cell/mL and left for complete adherence for 48 h. Cells were then incubated with L, L7G or FF at 20 μM, and SIMV at 10 μM, for 24 h. At the end of incubation time, cells were harvested using Trypsin-EDTA solution as described above, conveniently washed with cold PBS, centrifuged at 500 g for 5 min and supernatant was discarded. The remainder of the procedure was carried out in the fume hood. Cells were lysed using 175μL RNA lysis buffer supplemented with β-mercaptoethanol, and dispersed by successive pipetting. 350 μL of RNA dilution buffer were added and mixed by tube inversions before incubation at 70 °C for 3 min followed by centrifugation at 14000 g for 10 min at room temperature. The clear lysate obtained was transferred to a new eppendorf tube and mixed by pipetting with 200 μL of 95 % ethanol, the mixture obtained was then transferred to spin column assembly and centrifuged at 14000 g for 1 min. Columns were then washed with RNA wash solution containing ethanol followed by another centrifugation at 14000 g for 1 minute. Subsequently the DNase treatment solution, freshly prepared (5 μL 0.09 M $MgCl_2$, 5 μL DNase I enzyme in 40 μL yellow cure buffer), was added to membrane of column maintained for 15 min before the addition of DNase stop solution and centrifugation at 14000 g for 1 min, followed for two washes steps. Finally 100 μL nuclease-free water was added to spin column and recovered in a new tube by centrifugation at 14000 g for 2 min. RNA quantification was performed using NanoDrop ND-1000 spectrometer, and only samples with Abs_{260}/Abs_{280} ratio between 1.8 and 2.1 were considered. RNA samples were stored at -80 °C until further analysis

cDNA synthesis and quantitative Real Time – Polymerase Chain Reaction

The cDNA synthesis was performed using iScript™ cDNA Synthesis Kit (Bio-Rad), as template was used 1 μg of purified RNA, and volumes normalized with nuclease-free water. The complete reaction mix (RNA sample, 1x iScript reaction mix and iScript reverse transcriptase) was incubated for 5 minutes at 25 °C followed by 60 min at 42 °C

and for final 5 min at 85 °C, in a thermocycler MyCycler from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). The cDNA was then stored at -20°C.

Differences in gene expression were assessed by qRT-PCR. Quantitative gene expression analysis was performed using SYBR Green technology (SsoFast EvaGreen supermix) and CFX96TM Real-Time system from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Reaction solution was obtained with 10 μL of SsoFast EvaGreen supermix, plus 500 nM from each primer (forward and reverse). As template 1 μL of cDNA was used. In each experiment, all the samples were measured in duplicates, as well as controls without template. Samples were amplified using the following conditions: an initial denaturation step of 3 min at 95°C followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. As reference gene *β-actin* was used, and the genes tested were *SREBP-2*, *HMGCR*, *LDL receptor*, *ABCA1* and *CPT1*. Signals above 38 Cq were not considered, in order to reduce misleading results. The fold induction was determined using the ΔΔCq method as described in (Bookout and Mangelsdorf, 2003).

Statistical analysis

All the results are presented as means ± SEM. For statistical analysis One-way ANOVA followed by Dunnett's comparison test were performed using. GraphPad Prism 5.0 software (San Diego, CA, USA). Differences were considered significant when p value was $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ when compared with the control group.

3.3 - Results

In order to determine the concentrations of the flavones L and L7G, and SIMV and FF, as reference compounds, to be used in the subsequent tests using HepG2 cells, we performed a MTT reduction assay. In *Figure 3.2* we can see that incubation with L at 30 μM produces a decrease in cell viability of about 16 %, while L7G seems not to alter significantly the viability of HepG2 at none of the concentrations tested. Also, FF only at the highest concentration decrease cell viability by approximately 10 %. With regard to SIMV, at 20 μM caused a decrease in cell viability of around 25 %. We, therefore, decided to use L, L7G and FF at 20 μM, which allows a direct comparison of effects without

impact on cell viability. SIMV was used at 10 μM a more likely concentration to be obtain *in vivo* by patients undergoing treatment with SIMV(Bonn et al., 2002).

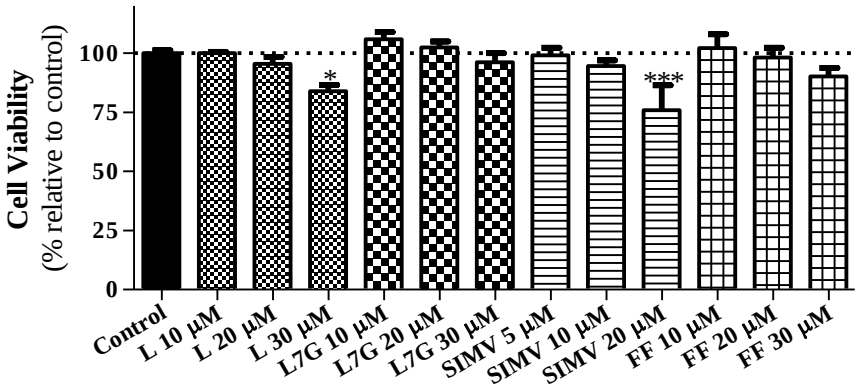


*Figure 3.2 – Effect on cell viability of HepG2 cells of L, L7G, SIM and FF, upon 48 h of incubation. HepG2 cells were exposed to different concentrations of the compounds in test, and after 48 h cell viability was assessed using MTT reduction assay. The results are presented as percentage relative to control: mean ± SEM of three independent assays. For statistical analysis One-way ANOVA followed by Dunnett's comparison test were performed, (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 when compared with the control group).*

With the purpose of evaluating if both L and L7G have the capacity to reduce cholesterol levels, cellular cholesterol was extracted using chloroform and quantified. Quantification of total cellular cholesterol (TCC) after 24 h of incubation with L, L7G or SIMV, produced no alterations in cholesterol content of cells, *Figure 3.3 A*. Taking into account that reduction of cholesterol in membranes may take longer than 24 h, a longer incubation period, 72 h, was used. As shown in *Figure 3.3 B*, even at 72 h, SIMV and flavones separately did not seem to alter TCC in a statistically significant manner. In fact, it was only when cells were co-incubated with both SIMV and the glycosylated form, L7G, that we were able to observe a reduction in cholesterol content (of about 28.6 % relatively to control cells).

To be sure that compounds with which the cells were incubated do not interfere with the kit used to quantify cholesterol, the kit was applied using as sample the concentrated stocks of L, L7G or SIMV. None of compounds resulted in absorbance higher than blank (dat not shown), demonstrating that the compounds did not interfere with the cholesterol quantification method.

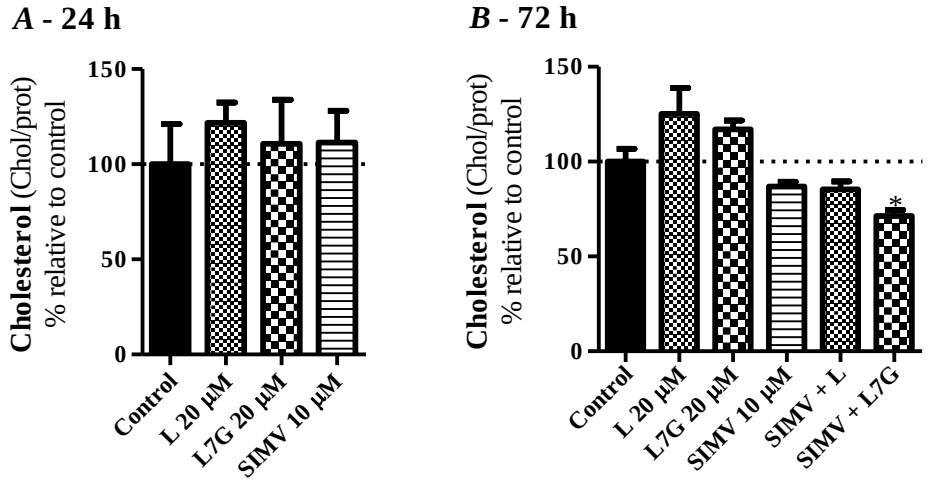


*Figure 3.3 – Quantification of total cellular cholesterol. A – HepG2 cells incubated with L, L7G or SIMV, for 24 h. B – HepG2 cells incubated with L and L7G alone or in co-incubation with SIMV. The results are presented as % relative to control. Values are mean ± SEM of three independent assays. For statistical analysis One-way ANOVA followed by Dunnett's comparison test, were perfomed, (*p ≤ 0.05 when compared with the control group).*

Cholesterol levels in cell are tightly controlled and SREBP-2 is a major modulator of cholesterol levels. When SREBP-2 is activated in the face of decreasing TCC. The activation (cleavage) of SREBP-2 induces *HMGCR* gene expression in order to compensate the decreased levels of cholesterol. We evaluated if L or L7G alone, and in combination with SIMV, were able to alter the levels of cleaved form. Considering that the activation occurs through a proteolytic process, western blot was used to analyse SREBP-2 levels, once it allows the detection of both the total form (around 125 kDa) and cleaved/active form (approximately 60 kDa). However, we were not able to obtain reliable signals with the antibody we had available. Thus in *Figure 3.4*, a representative immunoblot of the expression of cleaved SREBP-2 relative to β-actin, the loading control, and not total SREBP-2, is presented. The results show that L reduces SREBP-2 active form relatively to control (about 33.7 %), while incubation with L7G does not result in statistically significant effects. SIMV itself leads to a reduction of approximately 57.5 %, and, contrary to expectations, co-incubation of SIMV with L seems to attenuate SREBP-2 reduction while, when co-incubated with L7G the reduction of active form is about 60.0 % although not different from SIMV itself.

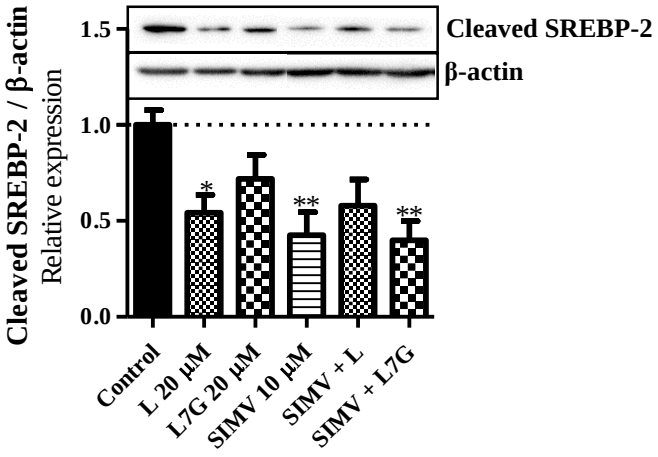


*Figure 3.4 – Comparison of SREBP-2 cleaved form levels in HepG2 cells with western blot. Cells were exposed to L, L7G or SIMV; and in co-incubation of SIMV with L or L7G, for 16 h. The results are presented as relative expression to control, using β-actin as loading control. Values are mean ± SEM of two independent experiences, with respective representative immunoblots. For statistical analysis One-way ANOVA followed by Dunnett's comparison test, were performed, (*p ≤ 0.05; **p ≤ 0.01 when compared with the control group).*

Subsequently, having in mind the effect on SREBP-2 activation we proposed to evaluate effects on gene expression of SREBP-2 target genes: *HMGCR* and *LDL receptor*, using qRT-PCR methodologies. Here we intended evaluate the effect of incubation of the compounds on SREBP-2 target genes, after the reduction of active SREBP-2 levels described, and compare with FF. In parallel, we tested if these compounds modify *ABCA1* gene expression, once that ABCA1 is a limiting transporter of cholesterol efflux, and HDL formation. *Figure 3.5* shows the effects of 24 h incubation with test compounds on *HMGCR* (*A*), *LDL receptor* (*B*) and *ABCA1* (*C*). The results show that expression of *HMGCR* was significantly decreased by L and SIMV, by 38.1% and 45.0 %, respectively. With regard to *LDL receptor*, another SREBP-2 target, none of the compounds had a significant effect, although its expression varies with the same tendencies that *HMGCR*. Also, expression of *ABCA1* was not altered. FF also did not affect any of the genes tested.

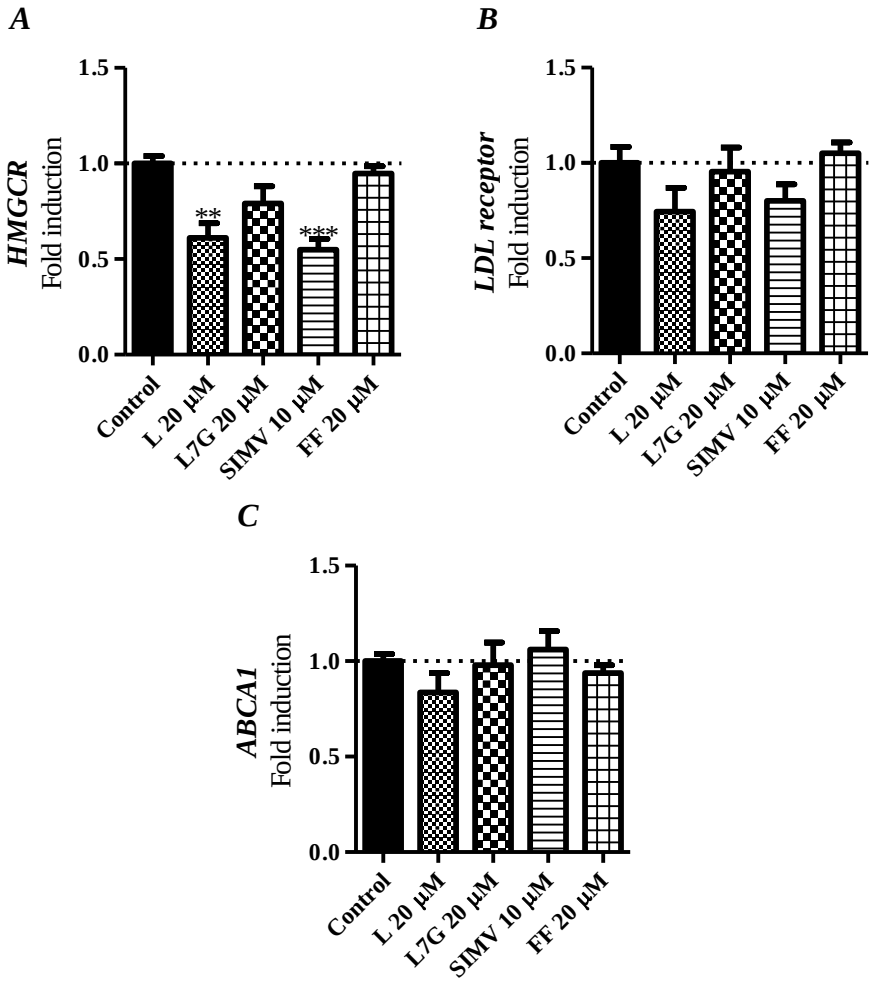


*Figure 3.5 – Analysis of HMGCR (A), LDLr (B) and ABCA1 (C) gene expression using qRT-PCR technology. Cells were exposed to L, L7G, SIMV or FF, for 24 h. The results are presented as fold induction relative to control conditions, using β-actin as endogenous control. Values are mean ± SEM, of three independent experiences. For statistical analysis One-way ANOVA followed by Dunnett's comparison test, were performed, (*p ≤ 0.05, ** p ≤ 0.01, ***p ≤ 0.001 when compared with the control group).*

Aiming to evaluate if the effects of these compounds comprises alterations in FA metabolism (β-oxidation) besides cholesterol metabolism, we analysed PPARα protein levels. *Figure 3.6* shows the alteration on protein levels relative to β-actin, the loading control, when compared to control cells. This result, of one experience only, suggests that both flavones, L and L7G, increased PPARα levels. SIMV also seem to increase PPARα, and although the co-incubation of either flavone with SIMV also increased PPARα, the effect of both compounds did not seem to be cumulative.

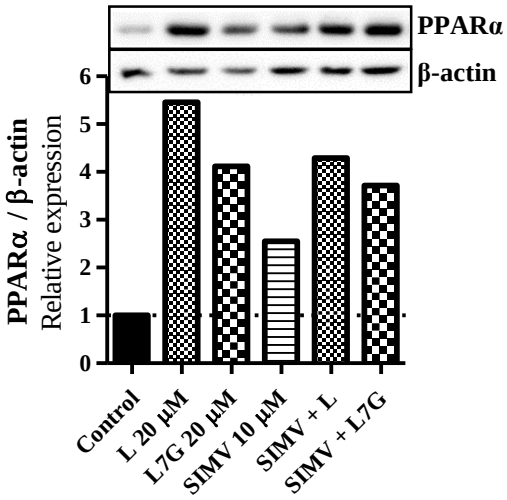


Figure 3.6 – Comparison of PPARα levels in HepG2 cells with western blot. Cells were exposed to L, L7G or SIMV; and in co-incubation of SIMV with L or L7G, for 16 h. The results are presented as relative expression to control, using β-actin as loading control, with respective immunoblots.

PPARα levels could be an indicator of β-oxidation, once it is a transcription factor for expression of genes essential in the process. The result we were able to obtain, although preliminary seems very promising. The antibody used is specific for human PPARα, (in fact HepG2 cell lysate is a positive control for him, however, we only were able to obtain proper signal in a unique experience with this samples). We, therefore, decided to analyse the *CPT1* gene expression, a target gene of *PPARα*, by qRT-PCR. FF was included in this experiment since it is a synthetic agonist of the ligand-activated transcription factor PPARα. The effect of the flavones, L and L7G, as well as SIMV and FF on gene expression of *CPT1* is shown in *Figure 3.7*.

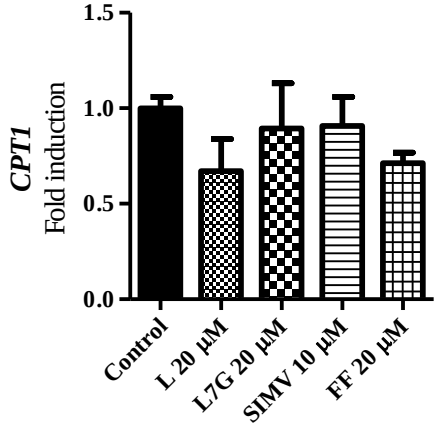


*Figure 3.7 – Analysis of CPT1 gene expression using qRT_PCR technology. Cells were exposed to L, L7G, SIMV or FF, for 24 h. The results are presented as fold induction relative to control conditions, using β-actin as loading control. Values are mean ± SEM of three independent experiences. For statistical analysis One-way ANOVA followed by Dunnett's comparison test, were performed, (*p ≤ 0.05, when compared with the control group).*

The transcription rate of *CPT1* was not significantly changed after 24 h of incubation with the L, L7G or SIMV. Unlike expected, since it is an agonist of PPARα, also FF did not changed *CPT1* gene expression.

As CPT1 is a mitochondrial enzyme fundamental in FA translocation into the mitochondria where β-oxidation occurs. The levels of CPT1 can be related to β-oxidation capacity. However, CPT1 activity can be controlled through its direct inhibition by malonyl-CoA, produced by ACC enzyme in its active (non-phosphorylated) state. ACC protein levels were assessed by western blot. The results of a preliminary experience are shown in *Figure 3.8*. Protein levels of phosphorylated-ACC relative to total ACC, after 16 h of incubation with L, L7G, SIMV or FF.

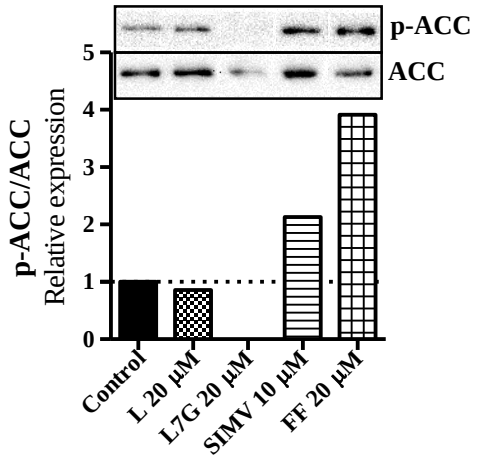


Figure 3.8 – Comparison of p-ACC protein levels relative to total ACC in HepG2 cells with western blot. Culture cells were exposed to L, L7G, SIMV or FF 20 μM, for 16 h. The results are presented as relative expression to control, with respective immunoblots.

These results suggest that L did not change p-ACC levels. While L7G dramatically reduced p-ACC. On the other hand, SIMV and FF seem to increase p-ACC at 16h of incubation.

3.4 - Discussion

Naturally occurring flavones such as Luteolin and Luteolin-7-O-Glucoside are widely present in fruits, vegetables and medicinal herbs, which could be easily introduced on our diet. Because of the variety of foods in which L and L7G are present, it seems easy to adapt our diet in order to increase the daily of L and L7G intake. This approach could be beneficial having into account the health promoting effects associated with these compounds (Azevedo et al., 2010; López-Lázaro, 2009). The control of lipid metabolism and

particularly the prevention of hypercholesterolemia and dyslipidaemia would constitute valuable prevention of metabolic diseases, including CVD and NAFLD incidence.

Our study showed that both L and L7G have effects on the control of cholesterol synthesis, in human liver cells. In addition, although preliminary results, L and L7G seem favour FA β-oxidation. The concentration range of test compounds used were chosen in agreement with the literature, and with physiological relevance (Bonn et al., 2002; Jiao, 2002; Lee et al., 2006).

The results show that, as SIMV, L and L7G did not change cholesterol levels in HepG2 cells, at 24 h of incubation. After 72 h, only the co-incubation of L7G and SIMV reduces cholesterol levels significantly, possible due to a cumulative effect. At 24 h the levels of TCC vary between 10 and 13 μg of cholesterol/mg protein, the values are in the same order of magnitude as TCC in HepG2, published by (Scharnagl et al., 2001).

The effect of reducing cholesterol synthesis in HepG2 was already reported using high doses of Artichoke extracts which had an inhibitor effect on cholesterol biosynthesis on HepG2 cells and rat hepatocytes (Gebhardt, 1998, 2002). The authors attributed the effect to L and L7G found in high concentrations in this plant, in rat hepatocytes some of the compounds more abundant in Artichoke extracts were tested, L (at concentrations between 175 and 350 μM) was the stronger inhibitor of the biosynthesis of neutral lipids from ^{14}C-acetate, L7G (at concentrations between 112 and 224 μM) presented a lower inhibitory capacity (Gebhardt, 1998). In HepG2 cells the inhibitory effect was enhanced when cells were concomitantly exposed to extracts and β-glucosidases, which cleaves glycoside from L7G, the most abundant form on extracts, increasing L concentration and potentiating the activity. According to the authors HepG2 cells do not seem to have endogenous β-glucosidase activity sufficient to fully convert L7G to L (Gebhardt, 2002). The inhibition of cholesterol synthesis by these extracts seems to be dependent of effects on HMGCR, although extracts did not directly inhibit these enzyme activity (Gebhardt, 1998). In fact, our own previous results show that L7G does not directly inhibit HMGCR, *in vitro*, at least not to a comparable extent to statins (Sá et al., 2015). Is important to note that the concentrations tested by (Gebhardt, 1998), were much higher than the concentrations used in the present study.

To investigate the possibility that the reduction of cholesterol through L7G plus SIMV was due to a gene transcription regulation of rate-limiting enzyme of cholesterol synthesis besides its inhibition, we analysed if these compounds affect the transcription factor, SREBP-2 activation, and its targets *HMGCR* and *LDL receptor* gene expression.

Our results, obtained by western blot analysis, show that, in fact, the co-incubation of L7G and SIMV leads to a reduction of cleaved SREBP-2 after 16 h, which is in line with the reduction of TCC observed with these compounds 72 h. In addition, the same effect was observed by incubation with L or SIMV alone. Interestingly, the co-incubation of L with SIMV did not reduced SREBP-2 cleavage, despite the individual effect. Our results do not show an increased activation of SREBP-2 by SIMV, as expected, but these may be explained by the fact that there was no effect on reduction of TCC levels due to exposure to the compounds used in the experiment. As it have been suggested that incubation of HepG2 with statins result in increase in cleaved form of SREBP-2 (Scharnagl et al., 2001). And, other studies show that SIMV administration results in up-regulation of *SREBP-2* and also *HMGCR* its gene target which could be due to the decrease in cholesterol levels induced by activity inhibition of HMGCR, leading to SREBP-2 activation and increasing its nuclear localization (Rayner et al., 2010).

The qRT-PCR analysis shows a reduction of *HMGCR* gene expression, in result to exposure to L and SIMV, which is congruent with the decrease in activation of its transcription factor. The levels of *LDL receptor* follow the same pattern. This response to L with a reduction of SREBP-2 activation and of *HMGCR* gene expression decrease in response to L, may be beneficial for NAFLD patients, since, increased levels of free cholesterol are known to be presented in NAFLD patients, dependent on *SREBP-2* induction, as well as *HMGCR* higher expression (Caballero et al., 2009).

Levels of *ABCA1* transcription are regulated by *SREBP-2* transcription because with *SREBP-2* expression the microRNA miR-33 is also transcribed which directly inhibits *ABCA1* translation and, consequently, limits cellular cholesterol excretion and reverse cholesterol transport (Allen et al., 2012). In our study both L and L7G did not alter *ABCA1* gene transcription.

PPARα protein analysis by western blot, albeit not definitive was indicative of its increased levels upon incubation with L, L7G or SIMV. It has been demonstrated that the presence of ligand of PPARα decreases its ubiquitination, increasing protein stability and inhibiting PPARα degradation which consequently should result in the up-regulation of its target genes (Blanquart et al., 2002). In order to verify if L and L7G act as PPARα agonists increasing target gene expression, we perform qRT-PCR analysis of CPT1. Gene transcription of *CPT1* was not significantly changed with incubation of L, L7G, SIMV or FF. However, since FF is a known agonist of PPARα activation, it was expected an increase in its target CPT1 (Blanquart et al., 2002).

However, it is important to note that CPT1 besides being regulated at gene transcription level, is also directly inhibited by the presence of malonyl-CoA. ACC is the enzyme responsible, in the FA synthesis pathway, for malonyl-CoA production. ACC is inactivated by post-transcriptional phosphorylation (Koo, 2013). The western blot analysis of p-ACC and total ACC showed that FF incubation at 16 h seems to increase levels of the inactive form of ACC: p-ACC, indicating an inhibition of FA synthesis pathway. L seems not have an effect at this level, while L7G seem to decrease p-ACC, although a complete disappearance of p-ACC band could be more dubious, in an analysis by western blot.

It is necessary to take into account that, the benefits of flavones, as other flavonoids, tend to be effective only through long term exposure. That is, the benefits of flavones seem to be dependent of successive regular exposures (Nijveldt et al., 2001). This explain why successive diet supplementation results in up-regulation of PPARα, in rats (Sá et al., 2015), an effect that can be related to the accumulation of the protein that we observed, in this study. Further research is required, however, to confirm effects of the consumption of food rich in these compounds on PPARα and conclude whether this strategy may be suitable for the promotion of FA β-oxidation, preventing FA accumulation and NAFLD (Gusdon et al., 2014; Koo, 2013).

Combination therapy with both statins (in these case atorvastatin) and fibrates (in these case FF), in patients with MetS and early NAFLD for 54 weeks seem to be effective in elimination of NAFLD symptoms, in about 70 % of the patients (Gusdon et al., 2014). Considering the cumulative effect of L7G with SIMV in cellular cholesterol reduction, as well as the reduction on SREBP-2 cleavage and the decreased *HMGCR* gene expression with L, it seems that the consumption of these flavones may result in similar effects to statins treatment. On the other hand, accumulation of PPARα protein: may result in similar effects of PPARα agonists such as fibrates. Nevertheless, more enlightening studies and more consistent results are needed, before the statin or fibrate like effects of these flavones (Sá et al., 2015), in human liver cells can be concluded.

Chapter 4 - General Conclusions and Future Perspectives

These work had a focus on two different features of lifestyle which can be adapted according: diet and physical activity. In addition, besides a source of calories, diet is also a source of micronutrients, such as polyphenols, flavonoids, such as the flavones L and L7G, which are associated with health improving properties. Therefore, a balanced diet and an improvement in physical activity may prevent the development of metabolic diseases, such as CVD, T2DM and NAFLD.

Part A of this study focuses on the effect of two different kinds of diet: standard diet (35 % of calories derived from fat) and high-fat diet (71 % of calories derived from fat). And their interaction with two different exercise regimens: voluntary physical activity, during all the experiment, and endurance training, which began only 8 weeks after the start of feeding with the diets. We aimed to analysed expression of transporters related to cholesterol absorption and secretion, and the main regulators of cholesterol synthesis using qRT-PCR. The levels of global DNA methylation were also analysed. It seems that a high-fat diet results in increased levels of global DNA methylation, and only voluntary physical activity counteract this effect. Neither diet nor exercise affected expression of *SREBP2* and *ABCA1*, in this experiment. Although, the consumption of a high-fat diet increased *ABCG5* expression, and similarly only voluntary physical activity reduces *ABCG5* expression for levels comparable to standard fed rats. This reduction on *ABCG5* expression, may impair sterol elimination, explaining the higher cholesterol levels, previously observed in high-fat diet rats with voluntary physical activity. The expression of *NPC1L1*, was not changed by diet, however both types of exercise reduced its expression in standard fed rats. But, in high-fat diet group, the incorporation of endurance training did not change *NPC1L1* expression. This reduction in *NPC1L1* by voluntary physical activity in both diets, and by endurance training in standard diet, shows that exercise may reduce cholesterol absorption.

Part B of this work characterizes the protective effects of the flavones, L and L7G, on liver cholesterol metabolism, *in vitro*. Since these flavones are abundant in fruits and vegetables, which consumption is easily added (or increased) in the human diet, and, may be preventive of metabolic diseases. We evaluated the effects of L and its glycosylated form, L7G, in human hepatocellular carcinoma HepG2 cells. We analysed the effects of both forms of the flavone because although L7G is the most abundant form in nature, it has been described that L is the one who is absorbed. We assessed the effect of these compounds in human liver lipid metabolism, comparing with pharmacological drugs: SIMV and FF. The combination of L7G and SIMV at 72 h seems to result in reduction of

total cellular cholesterol. Incubation with L reduced activator cleavage of SREBP-2 and, also decreased *HMGCR* gene expression. These results can indicate a statin-like effect of these flavones, in HepG2. Incubation with L or L7G lead to an increase in PPARα protein levels, which could suggest a similar effect to fibrates, although it is premature conclude about these effects in human cells.

Achieving a regulation of lipid metabolism would be essential as a preventive measure against metabolic diseases. The combination of these preventive factors may reduce the severity of developed metabolic diseases, and later, delay the need for pharmacological treatments. Maintaining a regular physical activity, reduce fat intake and increase consumption in fruits and vegetables, particularly, olives, celery, peppers, broccoli, oregano, and parsley. Which are rich sources of L and L7G, and because of that, may be a good help in the prevention of abnormal cholesterol metabolic related diseases.

More detailed studies on these issues are needed before definitive conclusions can be drawn.

In order to better understand the changes in global DNA methylation it should be useful compare gene expression of DNMT1, DNMT3A and DNMT3B, enzymes responsible for DNA methylation. Alterations in their expression levels may explain the increased observed in high-fat fed rats. In addition, taking into account that more than the global levels of methylation, what causes changes in gene expression, are the patterns of methylation of CpG in promotors region of specific genes, it would be important to analyse these specific patterns in *ABCG5* and *NPC1L1* gene promoters to define whether they are under epigenetic regulation. This analysis may be achieved by methylation-specific PCR. Similarly, it would be of great importance perform the same analysis in *MLH1* and *MGMT*, once they are under epigenetic regulation and their hypermethylation is associated with colorectal cancer development.

It should be interesting, design other similar experiment in which the diets constitute a source of cholesterol. It should also be interesting introduce both exercise regimens in the same moment of the experiment. In a future experiment it would be better, instead of perform the analysis in the whole colon tissue, utilize only the enterocytes, obtained by scraping the surface layer of the rat's colon, after animals sacrifice.

With regard to the *in vitro* liver lipid metabolism study, it will be necessary to confirm the alterations provoked by incubation with L or L7G on levels of PPARα protein, and compare them with FF. Additionally, besides the *CPT1* gene expression, it would be important analyse if the protein levels change in response to L and L7G, by a

western blot analyses. Since that were found differences in phosphorylated state of ACC, it would be interesting also analyse the phosphorylated state of AMPK which is responsible for the regulation of ACC inactivation. Further, in order to continue the analysis of flavones effect on lipid metabolism, it would be necessary analyse SREBP-1 activate/cleaved form levels in response to L and L7G, as well as, analyse if these flavones alter the gene expression of *FAS*, a target gene of SREBP-1.

Although the work performed did not respond to all the questions about how diet and physical activity influence lipid metabolism, and more research is needed, these work showed the health beneficial effect of continuous voluntary physical counteracting a high-fat diet, and further showed the potential of naturally occurring flavones, L and L7G, in the prevention of metabolic-related diseases.

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Supplementary Information

Table S.1 – Description of diets constituents of Lieber DiCarli diets.

	Standard diet	***High-fat diet***
Calories	(1 Kcal/mL)	(1 Kcal/mL)
Fat	35 %	71 %
Carbohydrate	47 %	11 %
Protein	18 %	18 %
Ingredients	**g/Kg**	**g/Kg**
Casein	41.40	41.40
L-Cystine	0.50	0.50
DL-Methionine	0.30	0.30
Corn Oil	**8.50**	**48.50**
Olive Oil	28.40	28.40
Safflower oil	2.70	2.70
Maltose Dextrin	**115.20**	**25.60**
Cellulose	10.00	10.00
Choline Bitartrate	0.53	0.53
Xanthan Gum	3.00	3.00
Vitamins Mix	8.75	8.75
Mineral Mix	2.50	2.50
Vitamins Mix	**g/Kg**	
Thiamin HCl	0.60	
Riboflavin	0.60	
Pyridoxine HCl	0.70	
Niacin	3.00	
Calcium Pantothenate	1.60	
Folic Acid	0.20	
Biotin	0.02	
Vitamin B12 (0.1 %)	10.00	
Vitamin A Acetate (500.000 IU/gm)	4.80	
Vitamin D3 (400.000 IU/gm)	0.40	
Vitamin E Acetate (500 IU/gm)	24.00	
Menadione Sodium Bisulfite	0.08	
p-Amino Benzoic Acid	5.00	
Inositol	10.00	
Dextrose	939.00	
Mineral Mix	**g/Kg**	
Calcium Phosphate, bibasic	500.00	
Sodium Chloride	74.00	
Potassium citrate, monohydrate	220.00	
Potassium Sulfate	52.00	
Magnesium Oxide	24.00	
Manganous Sulfate monohydrate	4.60	
Ferrous Sulfate heptahydrate	4.95	
Zinc Carbonate	1.60	
Cupric Carbonate	0.30	
Potassium Iodate	0.01	
Sodium Selenite	0.01	
Chromium Potassium Sulfate	0.55	
Sodium Fluoride	0.06	
Sucrose	117.92	

Table S.2 – Blood analysis after 17 weeks of treatment. Retrieved from (Gonçalves et al., 2014a).

	SS	***SV***	***ST***	***HS***	***HV***	***HT***
Cholesterol (g/L)	69.00 ± 2.41	58.33 ± 2.46	51.40 ± 3.14	77.51 ± 4.14	88.80 ± 8.51	72.66 ± 3.93
HDL (g/L)	35.00 ± 2.05	38.72 ± 2.05	34.36 ± 1.02	55.50 ± 3.00	59.20 ± 4.76	48.41 ± 2.51

The Serenity Prayer

God grant me the serenity
To accept the things I cannot change
Courage to change the things I can
And wisdom to know the difference

Reinhold Niebuhr

And help me to face the world!