Cláudia Alexandra Canhoto Batista

Licenciada em Farmácia

Long-term hypercaloric diet consumption exacerbates age-induced metabolic dysfunction: Beneficial effects of CSN denervation

Dissertação para obtenção do Grau de Mestre em Bioquímica para a Saúde

Orientadora: Prof.ª Doutora Sílvia Vilares Conde, Professora Auxiliar, NOVA Medical School, Faculdade de Ciências Médicas, UNL

Co-orientadora: Doutora Joana Sacramento, CEDOC – NOVA Medical School, Faculdade de Ciências Médicas, UNL

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All animal studies described in this thesis were carried out in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU). Experimental protocols were part of the projects that were approved by the NOVA Medical School|Faculdade de Ciências Médicas Ethics Committee (CEFCM) and by the Direção-Geral de Alimentação e Veterinária (DGAV).

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- Corpo carotídeo: um potencial alvo terapêutico para as doenças metabólicas.
- Carotid chemoreceptor neuromodulation for the treatment of type ii diabetes mellitus.

The present work originated the following communications:

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Poster communications in national meetings:

Joana F. Sacramento, **Cláudia C. Batista**, Bernardete F. Melo, Cláudia S. Prego, Sílvia V. Conde. (2019) O consumo de dieta hipercalórica a longo prazo agrava a disfunção metabólica induzida pela idade: efeitos benéficos da ressecção do nervo do seio carotídeo. 15th Portuguese Congress of Diabetes, Vilamoura, Portugal.

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Abstract

The increasing incidence of metabolic disorders, like insulin resistance, type 2 diabetes (T2D), dyslipidemia, non-alcoholic fatty liver disease (NAFLD), obesity and metabolic syndrome (MS), has reached epidemic proportions. Knowing that life expectancy is increasing worldwide and that ageing is associated with a high prevalence of these disorders, it is crucial to stop this epidemic.

The carotid bodies (CB) are peripheral chemoreceptors, classically defined as O_2 sensors, also implicated in energy homeostasis. Our previous work showed in young animals submitted to 25 weeks of hypercaloric diets that the abolition of the CB activity restored the insulin sensitivity and glucose tolerance. Knowing that age is associated with the development of insulin resistance, the general aim of this work was to investigate the impact of long-term hypercaloric diet consumption and the abolishment of CB activity, through the CB sensitive nerve resection, the carotid sinus nerve (CSN) on glucose homeostasis in older rats. Also, knowing that NAFLD is one of the core comorbidities associated with dysmetabolism, we have focus on liver metabolism. Aged rats were fed with high-fat high-sucrose (HFHSu) diet for 44 weeks and after this period half was submitted to CSN resection and the other to a sham surgery. Animals were followed-up during 9 weeks for insulin sensitivity, glucose tolerance, fasting plasma glucose, weight and fat variation. After a terminal experiment lipid profile, liver lipid content and hepatocellular analysis were assessed. Abolishment of the CB/CSN activity in old rats reduced weight gain, restored insulin sensitivity and glucose tolerance, decreased plasma triglycerides and had beneficial impact on liver function.

Concluding, this study demonstrated for the first time that the modulation of the CB activity might be a potential therapeutic intervention in age-induced and diet exacerbated metabolic dysfunctions, as well in liver-associated metabolic dysfunction in young and old animals.

Key words: Aging, carotid body, carotid sinus nerve resection, dyslipidemia, insulin resistance, liver, metabolic diseases, non-alcoholic fatty liver disease, sympathetic nervous system, type 2 diabetes.

Resumo

A crescente incidência de distúrbios metabólicos, como resistência à insulina, diabetes tipo 2 (T2D), dislipidemia, doença hepática gordurosa não alcoólica (NAFLD), obesidade e síndrome metabólica (SM), atingiu proporções epidêmicas. A expectativa de vida está a aumentar mundialmente e o envelhecimento está associado a uma alta prevalência desses distúrbios, sendo crucial parar esta epidemia.

Os corpos carotídeos (CB) são quimiorreceptores periféricos, classicamente definidos como sensores de O_2 implicados na homeostase energética. Trabalhos anteriores mostraram em animais jovens com 25 semanas de dietas hipercalóricas, que a abolição da atividade do CB restaurava a sensibilidade à insulina e a tolerância à glucose. Sabendo que a idade contribui para a resistência à insulina, o objetivo geral deste trabalho foi investigar em ratos velhos o impacto de dietas hipercalóricas perlongadas e abolição da atividade do CB, através da ressecção do nervo do seio carotídeo (CSN) na homeostase da glucose. Sabendo que a NAFLD é uma das principais comorbidades associadas ao dismetabolismo, focamos no metabolismo hepático. Ratos idosos foram alimentados com dieta hipercalórica (HFHSu) por 44 semanas, após esse período, metade foi submetido à ressecção do CSN e a outra à cirurgia *sham*. Os animais foram acompanhados durante 9 semanas, avaliando-se a sensibilidade à insulina, tolerância à glucose, glicemia em jejum, variação de peso e gordura. Após o procedimento terminal o perfil lipídico, conteúdo lipídico hepático e análise hepatocelular foram avaliados. A abolição da atividade CB/CSN em ratos idosos reduziu o ganho de peso, restaurou a sensibilidade à insulina e a tolerância à glucose, diminuiu os triglicerídeos plasmáticos e teve um impacto benéfico na função hepática.

Concluindo, este estudo demonstrou pela primeira vez que a modulação da atividade do CB pode ser uma potencial intervenção terapêutica em disfunções metabólicas induzidas pela idade, exacerbadas pela dieta, assim como dismetabolismo associado ao fígado em animais jovens e velhos.

Palavras-chave: Envelhecimento, corpo carotídeo, ressecção do nervo carotídeo, resistência à insulina, fígado, doenças metabólicas, doença hepática gordurosa não alcoólica, sistema nervoso simpático, diabetes tipo 2.

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Abbreviations

AACE	American Association of Clinical Endocrinology
AHA/NHLBI	American Heart Association/National Heart, Lung, and Blood Institute
APDP	Associação Protectora dos Diabéticos de Portugal
apoB	Apolipoprotein B
ATP	Adenosine triphosphate
AUC	Area under the curve
BAT	Brown adipose tissue
BMI	Body mass index
BP	Blood Pressure
CA	Catecholamines
CB	Carotid bodies
CE	Cholesterol-esters
CM	Chylomicrons
CSN	Carotid sinus nerve
CTL	Control
CVD	Cardiovascular disease
DHEAS	Dehydroepandrosterone sulphate
EDTA	Ethylenediaminetetraacetic acid
EGIR	European Group for the study of Insulin Resistance
FA	Fatty acids
FFA	Free fatty acids
FPG	Fasting Plasma Glucose
GLP1	Glucagon-like peptide 1
GLUT2	Glucose transporter type 2
GLUT4	Glucose transporter type 4
HDL	High-density lipoprotein
HF	High-fat
HFHSu	High-fat high-sucrose
HSu	High-sucrose

H&E	Hematoxylin & eosin
IDF	International Diabetes Federation
IGF-1	Insulin-like growth factor-1
IMM	Instituto de Medicina Molecular
IRS	Insulin receptor substrate
ITT	Insulin tolerance test
K_{ITT}	Constant rate for glucose disappearance
LDL	Low-density lipoproteins
MAPK	Mitogen-activated protein kinase
MS	Metabolic syndrome
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCEP:ATPIII	National Cholesterol Education Program: Adult Treatment Panel III
OCT	Optimal cutting temperature
OGTT	Oral glucose tolerance test
PI3K	Phosphatidylinositol 3-kinase
PKB/AKT	Protein kinase B
ROS	Reactive oxygen species
SD LDL	Small-dense LDL
SNS	Sympathetic nervous system
TG	Triglycerides
T2D	Type 2 diabetes
VLDL	Very low-density lipoprotein
WAT	White adipose tissue
WHO	World Health Organization

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1. Introduction

1.1. Metabolic Diseases

Metabolic diseases are any type of disorder that disrupts the normal body metabolism, and comprises a broad spectrum going from congenital to acquired conditions (Enns, 2019). Acquired metabolic diseases like obesity, type 2 diabetes (T2D), hypertension, insulin resistance, hyperglycemia, non-alcoholic fatty liver disease (NAFLD), hyperlipidemia and metabolic syndrome (MS) are the most common types and have reach an alarming incidence rate both in developed and developing countries (Heindel *et al.*, 2017; Hossain, Kawar and Nahas, El, 2007; Hotamisligil, 2010). These metabolic diseases are considered multifactorial disorders intertwine with each other, with increasing prevalence and incidence (Kassi *et al.*, 2011). Moreover, a systemic deterioration that resembles a longevity-related one is common in these metabolic diseases (Hotamisligil, 2010).

1.2. Metabolic Syndrome

The MS is a cluster of complex disorders and is considered a major public-health epidemic, with increasing socioeconomic impact.(Alberti, Zimmet and Shaw, 2005) MS comprises a group of metabolic abnormalities that increase the risk of coronary heart disease, other forms of cardiovascular diseases, obesity and T2D (Grundy, 2004; Kassi *et al.*, 2011). The MS' main risk factors include: obesity, insulin resistance, raised fasting plasma glucose, hypertension and atherogenic dyslipidemia (elevated serum triglycerides (TG) and apolipoprotein B (apoB), increased small low-density lipoproteins (LDL), and reduced levels of high-density lipoprotein (HDL)) (Grundy, 2016; Grundy *et al.*, 2005; Kassi *et al.*, 2011). Moreover, MS is also associated with other metabolic abnormalities, such as a chronic proinflammatory and prothrombotic state, NAFLD and obstructive sleep apnea (Kassi *et al.*, 2011).

Named originally "Syndrome X" by Reaven (1988), was later renamed MS. Multiple definitions have been suggested throughout the years and because of that different definitions of the disease where used to identify patients (Mancia *et al.*, 2010). Due to the multiplicity of definitions is very challenging to compare data from different studies

and to determine the prevalence of this epidemic disorder. Although, it is estimated that around 20-25% of the adults worldwide have MS, a number that continue to increase (Nolan *et al.*, 2017). Data from the National Center for Health Statistics, estimates that in the US 1 in each 3 has MS (National Center for Health Statistics, 2012).

Initially, insulin resistance was suggested as the common and key pathological feature of MS, as it contributes to the development of T2D and cardiovascular diseases (Reaven, 1988). But now the MS' definition was reviewed by several international organizations and expert groups and currently takes into account different parameters, such as, differences in population age, sex, genetic background, diet and physical activity (Alberti, Zimmet and Shaw, 2005; Devers, Campbell and Simmons, 2016; Krishnadath *et al.*, 2016; Novak *et al.*, 2013; Pan and Pratt, 2008).

The MS' most widely used definitions are from the National Cholesterol Education Program: Adult Treatment Panel III (NCEP:ATPIII) and the International Diabetes Federation (IDF) (Table 1.1), that focus mainly on waist circumference, which is an alternative measure of central obesity. While, the definitions from World Health Organization (WHO), European Group for the study of Insulin Resistance (EGIR) and American Association of Clinical Endocrinology (AACE) focused on insulin resistance (Cornier *et al.*, 2008; Kassi *et al.*, 2011). Additionally, the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) states that the criteria to identify MS is based in the presence of three or more of the five risk factors (Grundy *et al.*, 2005) (Table 1.1).

The MS, as stated before, is a term for a cluster of endogenous risk factors that increase the risk of developing other metabolic diseases, like T2D and obesity. MS can be seen as a vicious cycle that fuels itself, as its incidence increases with other metabolic diseases and its manifestation triggers other metabolic diseases as well as the prevalence of the previously established disorders (Girod and Brotman, 2003). The syndrome is not caused by a single factor but instead by an array of risk factors (Kassi *et al.*, 2011). MS proves to be very heterogeneous in the population, as it can be clinically manifested in a variety of ways, especially among different racial and ethnic groups. Thus, making it very challenging to create guidelines for its definition (Mancia *et al.*, 2010).

Clinically, the first approach to manage MS is based on lifestyle interventions aiming to lower hypertension, fasting plasma glucose, atherogenic dyslipidemia, abdominal obesity and insulin resistance. Usually, lifestyle changes are difficult to implement or are not enough to manage MS and there is the need to introduce a pharmacological approach (Grundy *et al.*, 2005; Thorp and Schlaich, 2015). Due to its multifactorial components and to its numbers and its increase additional research is needed to better understand the key pathological feature of MS.

Table 1.1 - International Diabetes Federation (IDF) definition for Metabolic Syndrome (MS).

For a person to be defined as having metabolic syndrome they must have: **• Central obesity*** **• Plus any two of the following four factors:**	
Raised Triglycerides	≥ 150 mg/dl (1.7 mmol/l) or specific treatment for this lipid abnormality
Reduced HDL Cholesterol	< 40 mg/dl (1.03 mmol/l) in males < 50 mg/dl (1.29 mmol/l) in females or specific treatment for this lipid abnormality
Raised blood Pressure (BP)	Systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg or treatment of previously diagnosed hypertension
Raised Fasting Plasma Glucose (FPG)	FPG ≥ 100 mg/dl (5.6 mmol/l) or previously diagnosed T2D If above 100 mg/dl or 5.6 mmol/l, OGTT is strongly recommended but is not necessary to define presence of the syndrome.

* Central obesity is defined as waist circumference with ethnicity specific values. If body mass index (BMI) is > 30 kg/m2, central obesity can be assumed and waist circumference does not need to be measured. In Europe, values for waist circumference are ≥ 94 cm in males and ≥ 80 cm in females. OGTT – Oral Glucose Tolerance Test.

1.1.1. Type 2 Diabetes Mellitus

According to the American Diabetes Association and WHO, the classification of diabetes mellitus comprises four clinical classes: type 1 diabetes, T2D, gestational diabetes and other particular types of diabetes duo to other specific causes (Reviriego, 2010; World Health Organization, 2006). The focus fall on the T2D as it is the most common type of diabetes, accounting for approximately 90% of all cases of the disease and considered the most common chronic diseases in the world (Bruno *et al.*, 2005; Holman, Young and Gadsby, 2015). The high prevalence of this disease is associated with changes in lifestyle, leaning towards reduced physical activity, sedentarism and increasing rates of obesity (Shaw, Sicree and Zimmet, 2010).

According with IDF statistics it is believed that in 2017, 425 million people worldwide had diabetes, a number that is expected to continue to increase and by 2045, it is estimated that 629 million people will have diabetes (Figure 1.1) (International Diabetes Federation - Diabetes atlas (8th edition), 2017). Furthermore, in the Portuguese population, it is estimated that 34.5% of the population, between the ages of 20-79 years, have diabetes or pre-diabetes and 44% of people with diabetes are unaware of their condition (Gardete-Correia *et al.*, 2010).

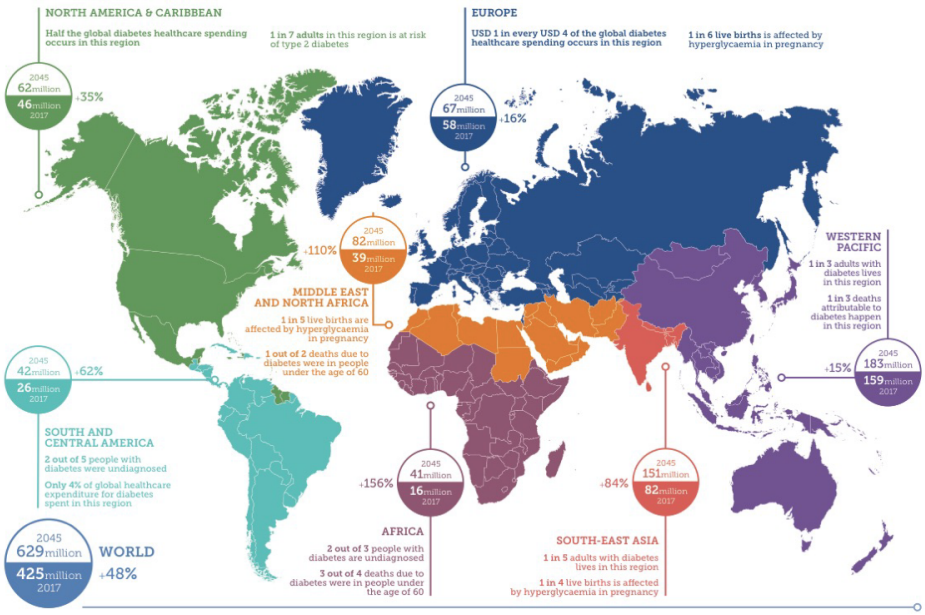

Figure 1. 1. Number of people with diabetes worldwide and per region in 2017 and 2045 (20-79 years) (IDF, 2017).

The early onset of this disease is called prediabetes and is characterized by a pancreatic β-cell dysfunction and a decrease in the insulin sensitivity, that starts before glucose changes are detectable (Tabák *et al.*, 2012). The risk factors for prediabetes are the same as the ones for T2D and include: overweight, aging, poor diet and excess of calories, reduced physical activity, smoking and family history (Forouzanfar, Alexander and Anderson, 2015; Vazquez *et al.*, 2007). However, people with prediabetes have a higher risk of developing T2D, yet not everyone with prediabetes will develop T2D.

Prediabetes is typically defined as blood glucose levels higher than normal, but bellow the diabetes diagnostic threshold for impaired glucose tolerance or impaired fasting glucose (Tabák *et al.*, 2012) (Table 1.2). In contrast, T2D is defined by higher blood glucose levels (≥ 126 mg/dl), being characterized by an intolerance to glucose and impaired fasting glucose, and also by a defect in the insulin secretion (β-cell dysfunction) and insulin action (insulin resistance) (Table 1. 2). Also, T2D is accompanied by dysregulation of carbohydrates, lipid and protein metabolism (DeFronzo *et al.*, 2015). Thus, the development of T2D from normal glucose tolerance to glucose intolerance and insulin resistance proves to be a continuous process, where several factors contribute to β-cell dysfunction, such as: aging, genetic abnormalities in incretin hormones (glucagon-like peptide 1 (GLP1) and gastric inhibitory polypeptide), lipotoxicity, glucotoxicity, reactive oxygen stress, hypersecretion of islet amyloid polypeptide and activation of inflammatory pathways (DeFronzo *et al.*, 2015).

Taking into account all the T2D aspects, including the presence of defects in both insulin secretion (β-cell dysfunction) and insulin action, it is understandable the interest in the identification and implementation of interventions to prevent or delay the diseases' onset (Kashyap and Defronzo, 2007).

Table 1.2 –Glycemic reference values from the Associação Protectora dos Diabéticos de Portugal (APDP).

Parameters	Hypoglycemia	Normal	Prediabetes	T2D
Fasting Plasma Glucose	< 70 mg/dl	70-100 mg/dl	100-126 mg/dl	≥ 126 mg/dl
2 Hours Post-prandial Glucose	< 70 mg/dl	70-140 mg/dl	140-200 mg/dl	≥ 200 mg/dl

1.1.1.1. Insulin

Insulin is a peptide hormone, organized in two polypeptide chains, A and B, connected by disulphide bridges, essential in regulation of carbohydrate and fat metabolism in the body.

In order to exert its action on glucose metabolism, insulin is synthesized and release by pancreatic β-cells, into the bloodstream. In the insulin sensitive tissues, like skeletal muscle, liver and fat tissue, insulin binds to its specific receptor, the insulin receptor. When insulin binds to its receptor it initiates the uptake of glucose from the blood into the insulin-sensitive tissue, as skeletal muscle and fat tissue (Kahn and White, 1988; Saltiel and Kahn, 2001). In the liver, insulin stimulates glycogen production and blocks glucose production, increasing the fatty acids (FA) content in the tissue. When the maximum capacity the FA in the liver is reached FA are released into the bloodstream and stored in the adipose tissue. Moreover, in the adipose tissue insulin inhibits the breakdown of TG into glycerol and FA (lipolysis) (Röder *et al.*, 2016).

The insulin receptor, a tetrameric glycoprotein, from the subfamily of the tyrosine kinases receptors, consists of four subunits: two α-subunit entirely extracellular, containing the insulin-binding domain, and two transmembrane β-subunit, expressing insulin-stimulated kinase activity (Guo, 2014; Pessin and Saltiel, 2000; Saltiel and Kahn, 2001). The binding of insulin to the α-subunit of the insulin receptor leads to the dimerization and formation of the complex, α2β2 in the cell membrane and consequently the autophosphorylation of the β-subunit, thus activating the insulin receptor tyrosine kinase (Bajaj and DeFronzo, 2003) (Figure 1.2). This activation results in the phosphorylation of members of insulin receptor substrate (IRS) family, namely IRS1 and IRS2, Gap-1 and Shc (Bajaj and DeFronzo, 2003).

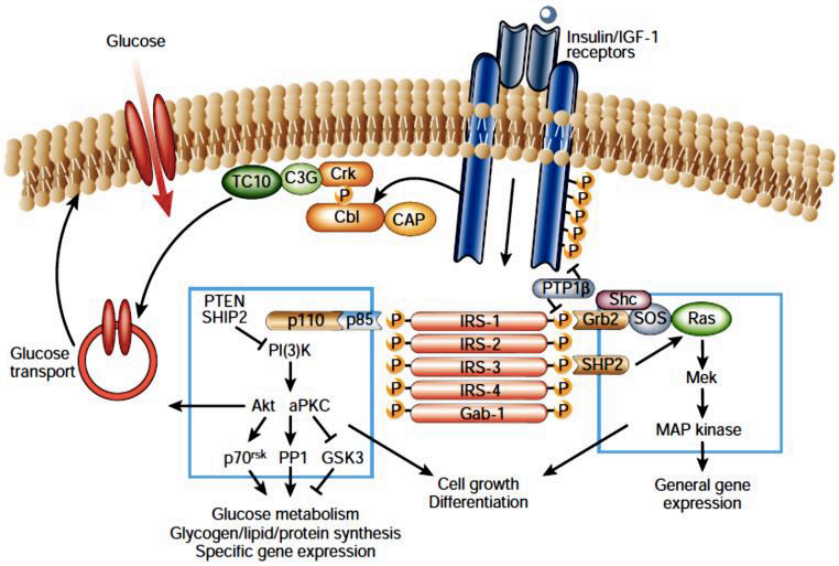

Figure 1. 2. Insulin signaling pathway. The increase in glucose levels stimulates the release of insulin from the pancreatic β-cells. Insulin binds to its insulin receptor, in insulin-sensitive tissues (adipose tissue and muscle), initiating a cascade of events that starts with phosphorylation of members of the insulin receptor substrate (IRS) family leading to the activation of phosphatidylinositol 3-kinase (PI3K), leading to the translocation of glucose transporter vesicles to the plasmatic membrane, thus enabling the uptake glucose. In the skeletal muscle and adipose tissue the glucose transporters are type 4 (GLUT4) (Saltiel and Kahn, 2001).

The phosphorylated IRS1 and IRS2 proteins bind to a heterodimeric enzyme, phosphatidylinositol 3-kinase (PI3K), which is composed by a p85 regulatory subunit and a p110 catalytic subunit (Bajaj and DeFronzo, 2003). Once PI3K is activated, it initiates a signaling pathway that activates protein kinase B (PKB/AKT), leading to the translocation of glucose transporter type 4 (GLUT4) vesicles to the plasmatic membrane of muscle cells and adipocytes and to the activation of glycogen synthase (Hajduch, Litherland and Hundal, 2001; Saltiel and Kahn, 2001). In the liver, the major glucose transporter is the glucose transporter type 2 (GLUT2), located in the membrane of the hepatocytes and believed to be linked to the insulin receptor (Eisenberg *et al.*, 2005). GLUT2 can transport bi-directionally glucose but also other sugars, like, galactose, mannose, fructose and glucosamine (Colville *et al.*, 1993; Karim, Adams and Lalor, 2012; Leturque, Brot-Laroche and Gall, Le, 2009; Zhao and Keating, 2007). In the fasting state, the liver produces glucose through glycogenolysis and

gluconeogenesis preceding to its release via GLUT2 (Karim, Adams and Lalor, 2012; Leturque, Brot-Laroche and Gall, Le, 2009). In the fed state, the rise of glucose and insulin levels inhibit hepatic glucose production, which leads to a decrease in GLUT2 mediated release and the internalization of the hepatocyte GLUT2 and the insulin receptor together into endosomes (Karim, Adams and Lalor, 2012; Leturque, Brot-Laroche and Gall, Le, 2009).

On the other hand, insulin also stimulates the mitogen-activated protein kinase (MAPK) signaling pathway through Grb2/Sos and ras, which is involved in gene expression, cell growth and differentiation (Cusi *et al.*, 2000) (Figure 1.2). Also, MAPK is involved in the regulation of hepatic metabolism (Kyriakis and Avruch, 2001; Tang *et al.*, 2014). In humans, the expression of p38δ MAPK has been shown to be increased in the liver of obese patients with NAFLD. So, MAPKs might drive hepatic metabolic dysfunction (Tang *et al.*, 2014). Although this mechanism is not yet fully understood, p38-MAPK signaling, activated by oxidative stress, is thought to be associated with diminished insulin-dependent stimulation of insulin signaling and glucose transport activity (Lawan and Bennett, 2017).

1.1.1.1.1. Insulin Signaling Defects in Type 2 Diabetes and Insulin Resistance

T2D is characterized by a defect on insulin action, where the insulin-sensitive tissues progressively loss the response to insulin, causing insulin resistance (Ye, 2013). Even though, the actual insulin resistance mechanisms remain unknown, several factors are linked with its manifestation, such as: obesity, inflammation, hyperinsulinemia, lipotoxicity /hyperlipidemia, stress, aging, fatty liver and hypoxia (Ye, 2013). The majority of these factors are deeply connected with obesity and aging, these making them the major risk factors for insulin resistance (Ye, 2013).

Although the insulin resistance mechanisms remain unclear the most expectable cause of its origin would be a downregulation of insulin receptors due to a generalized hyperinsulinemia, resulting from the increased insulin secretion from the beta cells (Grundy, 2016). This insulin overproduction leads to an increase in plasma insulin levels in fasting conditions. Independently of which appears first, hyperinsulinemia

appears to perpetuate the insulin resistance (Shanik *et al.*, 2008). Some studies demonstrated a connection between insulin resistance and the downregulation of insulin receptors (Molina *et al.*, 1989), others had shown that insulin binding to its receptors was normal in obese and lean diabetic individuals (Caro *et al.*, 1987; Kashiwagi *et al.*, 1983). In light of these studies, another hypothesis for insulin resistance based on a possible defect on insulin signaling in T2D, through a reduction of the insulin-stimulated tyrosine kinase activity, thus impairing the phosphorylation of IRS and activation of PI3K has emerged (Cusi *et al.*, 2000; Krook *et al.*, 2000; Saltiel and Kahn, 2001).

1.1.1.2. Glucose Homeostasis

Glucose is the main source of fuel for energy metabolism. Glucose homeostasis is ensured by a balance between glucose absorption from the intestine, glucose production by the liver and glucose uptake and metabolism by the peripheral tissues (Saltiel and Kahn, 2001).

The plasma glucose levels, in healthy people, are within the range of 4 and 7 mM, despite periods of fasting or feeding. After an overnight fast (10-12h), the majority of total glucose disposal takes place in the brain, which consumes around 50% of all glucose available. Approximately, 25% of glucose uptake takes place in the liver and gastrointestinal tissues. The remaining 25% is used mainly by the muscle, and in a lesser extent by the adipose tissue (DeFronzo, 2004). Additionally, the majority of glucose endogenous production is hepatic, approximately 85%, by glycogenolysis and gluconeogenesis. The kidneys contribute with 15% overall production (Gerich *et al.*, 2001; Magnusson *et al.*, 1992).

After glucose ingestion (Figure 1.3), a peak in plasma glucose concentration stimulates insulin release by the pancreatic β-cells. The resultant hyperinsulinemia and hyperglycemia stimulate the uptake of glucose by the liver, skeletal muscle and adipose tissue (Arner, 2005; Röder *et al.*, 2016). The influx of lipids into the adipose tissue leads to a decrease in lipolysis, with the inhibition of the TG hydrolysis into FA and glycerol (Arner, 2005). This inhibition leads to a decline in the plasma level of free fatty acids (FFA). Meanwhile, the uptake of glucose in the liver causes an inhibition of

hepatic glucose production (gluconeogenesis) together with the increase in glycogen production (Bergman, 2000; Boden and Shulman, 2002; DeFronzo, 2004; Defronzo *et al.*, 1985; Saltiel and Kahn, 2001). The glycogen production increases the FA content in the liver, which has a limited volume to store these lipids and when it can no longer hold the FA these are released into the bloodstream as FFA, that are later stored in the adipose tissue for posterior usage (Röder *et al.*, 2016).

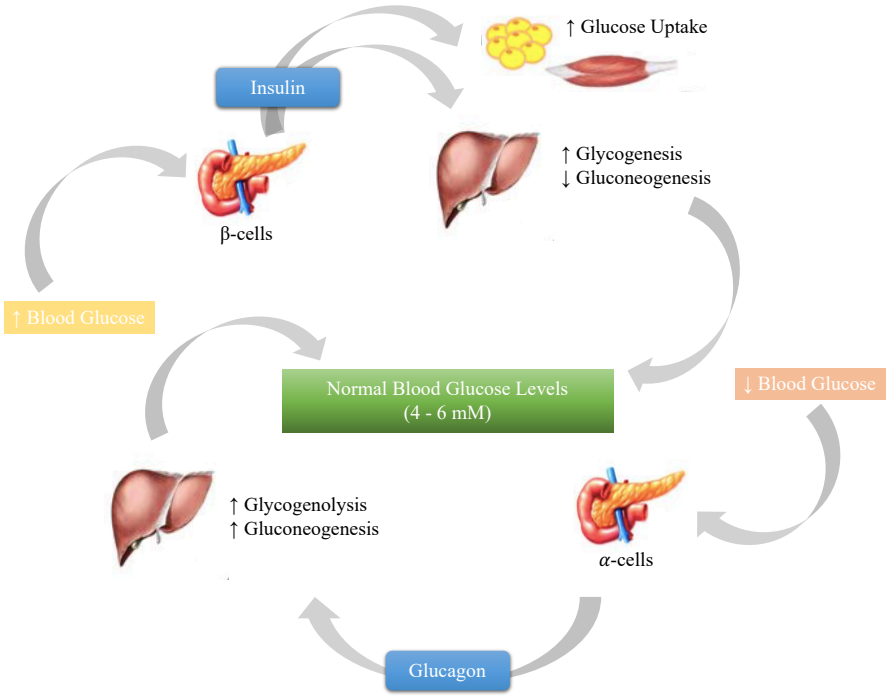

Figure 1. 3. Insulin and glucagon effects on glucose homeostasis. After food ingestion, the glucose levels increase, promoting the release of insulin from the pancreatic β-cells, which leads to the decrease in hepatic glucose production and the increase in muscle and adipose tissue glucose uptake. After an overnight fast, the blood glucose levels decrease, promoting the release of glucagon from the pancreatic α-cells, which leads to the hepatic glucose production, by increasing glycogenolysis and gluconeogenesis (adapted from Röder *et al.*, 2016).

Glucagon also plays an important role in the glucose homeostasis regulation (Figure 1.3). During fasting, approximately half of the total hepatic glucose output is dependent on the maintenance of normal basal glucagon levels (Cherrington, 1999). Whereas, after glucose ingestion, α-cell production of glucagon is inhibited contributing to the suppression of hepatic glucose production (DeFronzo, 2004).

Plasma glucose levels are maintained within the normal range (4-6 mM) by the action of two hormones released by the pancreas: insulin, released by the β-cells and glucagon, released by α-cell. The balance of these hormones plays a vital role in the regulation of the glucose homeostasis (Röder *et al.*, 2016).

1.1.1.2.1. Glucose Homeostasis Defects in Type 2 Diabetes

In T2D, the insulin sensitive tissues, such as the muscle, liver and adipose tissue, are characterized by defects in insulin sensitivity. Consequently, in T2D patients, after glucose ingestion, an increase in hepatic glucose production occurs, even in the presence of high levels of plasma insulin, demonstrating an hepatic resistance to the insulin action (Firth *et al.*, 1986). Studies have showed evidence that progression to glucose intolerance is associated with a prior development of insulin resistance (Figure 1.4.) (DeFronzo, Bonadonna and Ferrannini, 1992). The progression to glucose intolerance results from a defect in the β-cell, as the cells cannot maintain its previously rate of insulin secretion in response to high values of glucose (DeFronzo, 2004).

In the adipose tissue, insulin can no longer fully inhibit lipolysis, leading to an increase in FFA levels, resulting in skeletal muscle and liver's insulin resistance and impaired insulin secretion (Bajaj and DeFronzo, 2003; Boden and Chen, 1995; Kashyap *et al.*, 2003). Hence, the increase in FFA ultimately leads to an increase in hepatic glucose production and impaired skeletal muscle's glucose uptake (Boden *et al.*, 2001; Roden *et al.*, 1996).

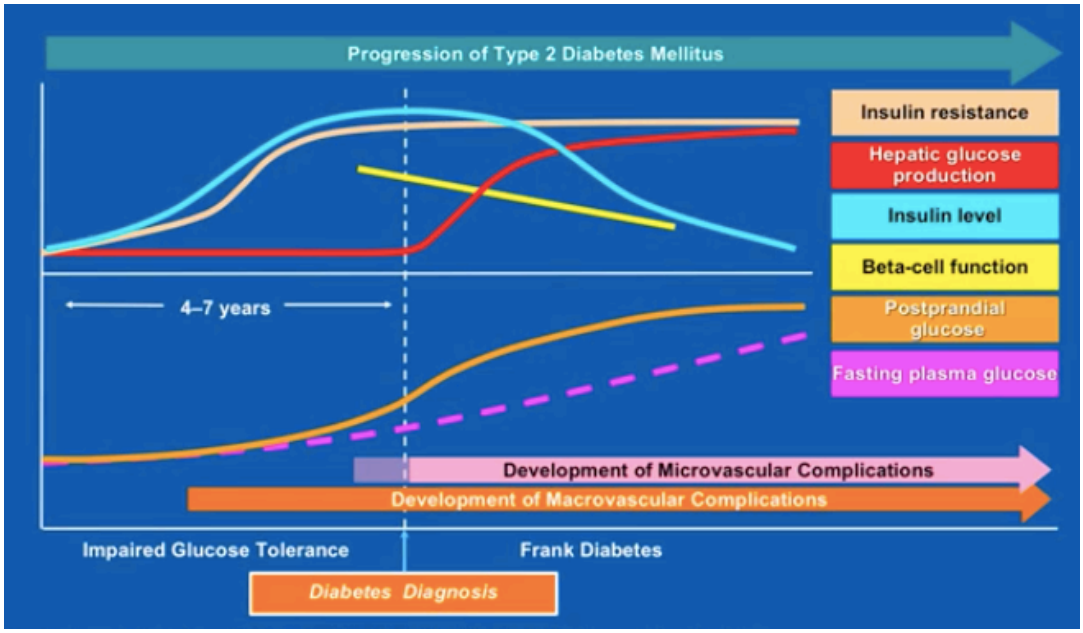

Figure 1. 4. Natural history of type 2 diabetes. The prediabetic state of impaired glucose tolerance is characterized by increasing insulin resistance, compensatory hyperinsulinemia, and mild postprandial hyperglycemia. Here, the fasting blood glucose levels are maintained in near normal ranges. Also, macro and microvascular complications start to arise. The β-cell then begins to fail, resulting in higher postprandial glucose levels and, with further loss of insulin secretory capacity and impaired glucorecognition, fasting blood glucose levels and hepatic glucose production increase (Ramlo-Halsted and Edelman, 1999).

1.1.2. Non-Alcoholic Fatty Liver Disease

NAFLD is seen as the hepatic manifestation of the metabolic diseases, recognizing insulin resistance as a key factor in the genesis of the disease (Vanni *et al.*, 2010). In contrast to the MS, the definition of NAFLD includes only one component: liver fat content > 5–10% per liver weight in the absence of excess alcohol consumption or any other liver disease (Vanni *et al.*, 2010). Despite of having only one component, the pathogenesis of NAFLD can be influenced by several factors, such as, increases in dietary fat supply or even genetic factors (Vanni *et al.*, 2010).

NAFLD compromises a broad disease spectrum from simple steatosis (non-alcoholic fatty liver, NAFL) to non-alcoholic steatohepatitis (NASH) and cirrhosis (Kotronen *et*

al., 2007; Neuschwander-Tetri and Caldwell, 2003) (Figure 1.5). The early stage of the disease is characterized by a benign and reversible state of simple steatosis (fat accumulation). Whereas, NASH is defined by an irreversible hepatocyte injury, presence of inflammation and/or fibrosis which can progress into cirrhosis, liver failure and hepatocellular carcinoma (Vanni *et al.*, 2010).

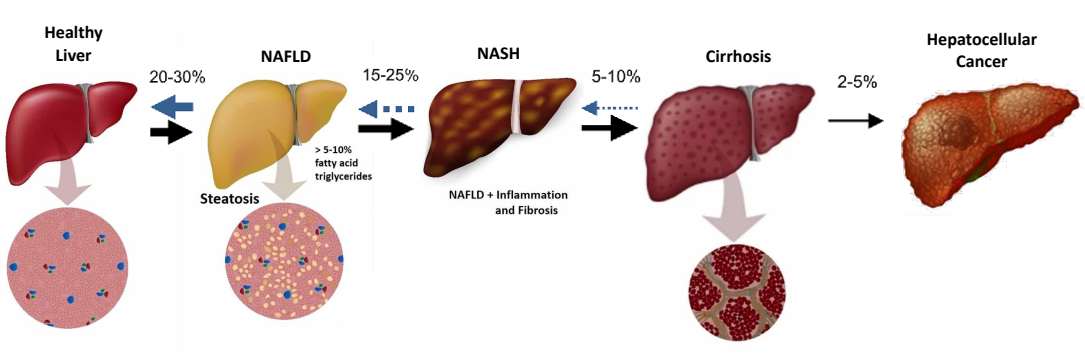

Figure 1.5. Schematic progression of non-alcoholic fatty liver disease (NAFLD) and estimated prevalence of the disease stages (adapted from Alwahsh *et al.,* 2016).

Approximately 10–25% of all patients with NAFLD develop NASH, although the evolution of steatosis to steatohepatitis remain unclarified (Day, 2005; Harrison, Torgerson and Hayashi, 2003). The "two-hits" model proposed by Day and James provide a pathophysiological foundation to the progression of liver damage, suggesting that the reversible deposition of TG ("first hit") leads to metabolic and molecular alterations that sensitize the liver to the "second hit", usually referred to as oxidative stress and cytokine-induced liver injury (Day and James, 1998). The liver is overwhelmed by the "second hit", leading to the irreversible hepatocyte injury seen in NASH, like lipid peroxidation, induction of inflammatory cells and fibrogenesis with extracellular matrix deposition (Vanni *et al.*, 2010).

NAFLD is a pathology that involves other organs besides the liver (Figure 1.6). For example, the muscle contributes to the pathology as when it becomes insulin resistant, glucose uptake in the muscle decreases and therefore the levels of circulating glucose are unable to decrease (Vanni *et al.*, 2010; Yki-Järvinen, 1993). Whereas, the adipose tissue contributes with an increase in the FFA. The lipolysis is not adequately suppressed by insulin, which leads to an increased FFA content, the main source of

hepatic TG in NAFLD (Vanni *et al.*, 2010; Yki-Järvinen, 1993). These factors together with liver´s overproduction of glucose, despite fasting hyperinsulinemia, contributes to the increase of fat deposition in the liver (Vanni *et al.*, 2010; Yki-Järvinen, 1993). The progressive increase in hepatic fat (hepatosteatosis) that leads to NAFLD, contributes for the impaired ability of insulin to inhibit gluconeogenesis (Bugianesi *et al.*, 2005; Seppälä-Lindroos *et al.*, 2002). In fact, the hepatic insulin resistance increases the plasma glucose concentrations, stimulating insulin secretion and driving the vicious cycle of hyperinsulinemia and hyperglycemia (Juurinen *et al.*, 2007; Vanni *et al.*, 2010).

The liver is the major organ for lipid distribution. The hepatic ability to store fat is limited and the lipid excess can be oxidized, but it is mainly released as very low-density lipoprotein (VLDL) (Kotronen *et al.*, 2008; Taskinen, 2005; Vanni *et al.*, 2010). So, after a meal, lipids are transported from the gut into the bloodstream in the form of chylomicrons (CM) and stored in the liver (Figure 1.6), where they are processed and assembled with apoB to form VLDL (Adiels *et al.*, 2006, 2007; Fabbrini *et al.*, 2008). One of insulin actions is to restrain VLDL production. However, in cases of hepatic insulin resistance, insulin is not able to inhibit VLDL production, leading to the overproduction of triglyceride-rich VLDL by the liver in the fasting state and during hyperinsulinemia (Adiels *et al.*, 2006, 2007; Fabbrini *et al.*, 2008; Lewis *et al.*, 1993). Consequently, hepatic insulin resistance could lead to hypertriglyceridemia (Kotronen *et al.*, 2008; Taskinen, 2005; Vanni *et al.*, 2010).

Studies suggest that hepatic fat accumulation is involved in the pathogenesis of T2D and that hyperinsulinemia might be a consequence of NAFLD (Juurinen *et al.*, 2007; Kotronen *et al.*, 2008). Although, many studies search to better understand the key pathological feature of NAFLD, still no current therapies exist for NAFLD or NASH (Vanni *et al.*, 2010).

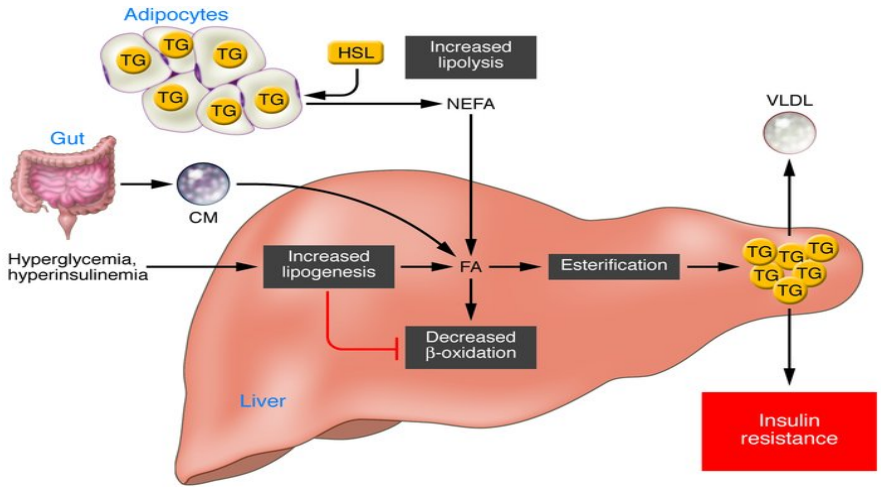

Figure 1. 6. Pathogenesis of Non-Alcoholic Fatty Liver Disease (NAFLD). Different sources of fatty acids contribute to the development of fatty liver. Under conditions of insulin resistance, insulin does not adequately inhibit hormone-sensitive lipase (HSL), and lipolysis in white adipose tissue is not suppressed. Therefore, peripheral fats stored in adipose tissue flow to the liver by way of plasma free fatty acids (FFAs). Dietary fatty acids are also taken up by the liver through the uptake of intestinally derived chylomicron (CM). In addition, the combination of elevated plasma glucose (hyperglycemia) and insulin concentrations (hyperinsulinemia) promotes *de novo* fatty acid synthesis (lipogenesis) and impairs β-oxidation, thereby contributing to the development of hepatic steatosis. After the esterification step (conversion of fatty acids into triglycerides (TG)) TG can then be stored as lipid droplets within hepatocytes or secreted into the blood as very low-density lipoproteins (VLDL) (Postic *et al.,* 2008).

Dyslipidemia is strongly associated with metabolic diseases, like MS, T2D, obesity and NAFLD (Katsiki, Mikhailidis and Mantzoros, 2016; Klop, Elte and Cabezas, 2013; Mooradian, 2009). A core characteristic of dyslipidemia is insulin resistance, which influences the increase in FFA and the deregulation of the liver and adipose tissue lipid metabolism (Katsiki, Mikhailidis and Mantzoros, 2016). The erratic lipolysis in the adipose tissue leads to the release of FFA that are converted to TG in the liver, resulting in hypertriglyceridemia. This shows how metabolic diseases are connected to each other, through the disturbance of lipids metabolism in the body (Blaton, Korita and Bulo, 2008).

In insulin resistant patients, the increased supply of glucose (Figure 1.7.) results in an increase in FFA released from insulin-resistant adipocytes into bloodstream that are stored in the liver (Frayn, 2001; Mooradian, 2009; Taskinen, 2003). This efflux of FA into the liver elevates TG production leading to the increase of apoB and VLDL (Frayn, 2001; Mooradian, 2009; Taskinen, 2003). The overproduction of VLDL drives the reciprocal exchange of FA: cholesterol-esters (CE) are transferred to VLDL and chylomicron remnants particles and TG are transferred to LDL particles, to form small-dense LDL (SD LDL), and to HDL particles. These dense particles are well known for their high atherogenic potential (Blaton, Korita and Bulo, 2008; Frayn, 2001; Mooradian, 2009; Taskinen, 2003). The increased number of VLDL particles and TG drive the increase of SD LDL particles, that can promote a slight increase of LDL particles and a decrease HDL levels (Chatrath, Vuppalanchi and Chalasani, 2012; Klop, Elte and Cabezas, 2013; Mooradian *et al.*, 2008; Mooradian, Haas and Wong, 2004; Wang and Peng, 2011).

In MS, similarly to obesity, cardiovascular disease (CVD), NAFLD and T2D, dyslipidemia is very commonly diagnosed, thus making it of vital importance in the vicious cycle of metabolic diseases (Chatrath, Vuppalanchi and Chalasani, 2012; Katsiki, Mikhailidis and Mantzoros, 2016; Manjunath *et al.*, 2013).

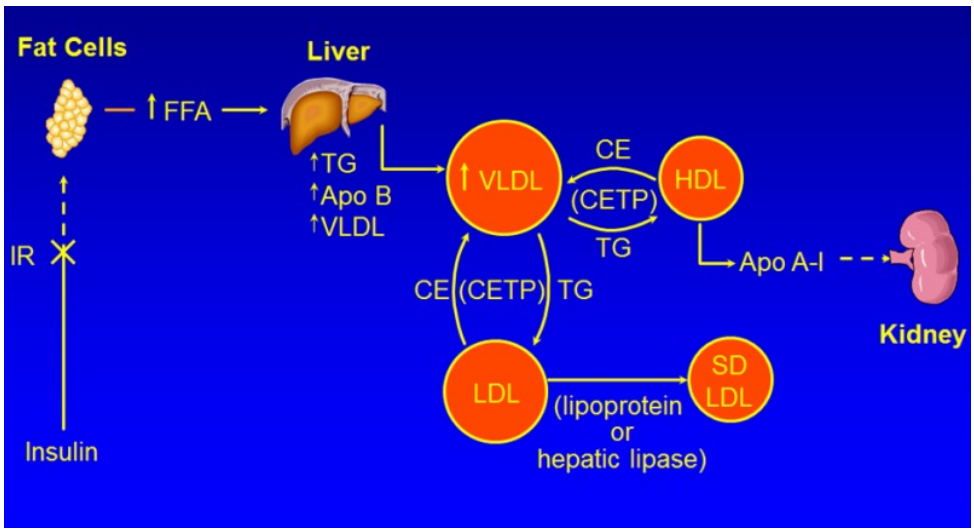

Figure 1. 7. Role of insulin resistance in T2D dyslipidemia. Insulin resistant cells release FFA leading to an increased flux into the liver which promotes TG production and secretion of apolipoprotein B (ApoB) and VLDL. Cholesteryl ester transfer protein (CETP) promotes the exchange from VLDL-transported triglyceride to high-density lipoprotein (HDL)-transported cholerteryl ester (CE), which results in increased amounts of both atherogenic cholesterol-rich VLDL remnant particles and triglyceride-rich, cholesterol-depleted HDL particles (Feingold *et al*, 2000).

1.3. Sympathetic Nervous System and Metabolic Dysfunction

The sympathetic nervous system (SNS) is an important component of the autonomic nervous system. Sympathetic outflow is driven by a network of neurons located in the rostral ventrolateral medulla. These neurons provide excitatory output to preganglionic neurons that innervate several target organs through postganglionic sympathetic fibers (Thorp and Schlaich, 2015).

The SNS plays a major role in the maintenance of the organism's homeostasis, being influenced by several environmental and genetic factors (Conde *et al.*, 2014; Tentolouris, Liatis and Katsilambros, 2006). SNS is responsible for controlling metabolic processes through regulation of the resting metabolic rate and thermogenesis, which leads to variations of energy states, carbohydrate consumption, and hyperinsulinemia levels (Thorp and Schlaich, 2015). The activation of sympathetic nerves innervation in the liver, pancreas, skeletal muscle and adipose tissue can also produce acute catabolic responses, like glycogenolysis and lipolysis (Thorp and Schlaich, 2015).

Due to the role of SNS activity in energy balance as well as several metabolic processes, a sympathetic overactivity might be associated to a variety of metabolic disorders, such as insulin resistance, T2D, hypertension, dyslipidemia, NAFLD and obesity (Conde *et al.*, 2014; Esler *et al.*, 2006; Grassi *et al.*, 2005, 2007; Huggett *et al.*, 2003; Kahn and Flier, 2000; Kobayashi *et al.*, 2010; Mancia *et al.*, 2007; Tentolouris, Liatis and Katsilambros, 2006). Moreover, there is substantial evidence supporting that the SNS is exceedingly active in individuals with the MS and its key metabolic alterations, central obesity and insulin resistance (Thorp and Schlaich, 2015).

In fact, the SNS has been described as one of the links between the presence of metabolic diseases and the development and progression of insulin resistance, as several components of the MS are associated with an increase in sympathetic activity and deeply connected to a decrease in insulin sensitivity (Bin-Jaliah, Maskell and Kumar, 2004; DeFronzo and Tripathy, 2009; Gallego-Martin *et al.*, 2012; Mancia *et al.*, 2007; Manjunath *et al.*, 2013; Sacramento *et al.*, 2018; Tsioufis *et al.*, 2007). It has been shown that the SNS can reduce insulin sensitivity by changing hemodynamic parameters, like vasoconstriction, making it more difficult for insulin to reach its target.

Moreover, the SNS can directly and indirectly favor the appearance and progression of organ damage, which is part of the clinical onset of the MS (Mancia *et al.*, 2007).

In metabolic diseases the increase in sympathetic activation can come from one of two ways, directly by the sympathetic nerve endings or indirectly by the circulating catecholamines (CA) (epinephrine and norepinephrine) (Conde *et al.*, 2014; Tentolouris et al. 2006). CA modulate metabolism to increase blood glucose levels by stimulating glycogenolysis in the liver, increased glucagon secretion and decreased insulin secretion from the pancreas, and increased lipolysis in adipose tissue. (Conde *et al.*, 2014; Tentolouris, Liatis and Katsilambros, 2006) Therefore, by a direct or indirect action, the SNS is associated with hyperinsulinemia, hyperleptinemia, increased FFA, inflammation, fatty liver and obesity, yet the mechanisms behind it remains to be clearly understood (Conde *et al.*, 2014; Lambert *et al.*, 2010). Blunted sympathetic responsiveness to glucose and elevated arterial noradrenaline levels are evidence for sympathetic overactivity, suggesting profound disturbances in central sympathetic nerve activity in metabolic diseases (Thorp and Schlaich, 2015).

Several pathophysiological mechanisms link SNS overactivity with the MS and its core components like central obesity and insulin resistance. The ongoing discussion as to whether sympathetic overactivity is a consequence or a cause of metabolic dysfunctions (Lambert *et al.*, 2010; Thorp and Schlaich, 2015). As, evidence suggest that SNS overactivity is important both in the initiation and the maintenance of metabolic abnormalities seen in the MS (Thorp and Schlaich, 2015).

1.4. Carotid Body

The carotid bodies (CBs) are small paired organs, located bilaterally at the bifurcation of the common carotid artery (Atanasova, Iliev and Lazarov, 2011; Gonzalez *et al.*, 1994) (Figure 1.7.). They measure approximately 2 mm in humans and less than 1 mm in rats (Atanasova, Iliev and Lazarov, 2011). The CBs are capable of sensing and responding to changes in arterial blood gases such as hypoxia (low O_2 levels), hypercapnia (high CO_2 levels), and acidosis (low blood pH) (Gonzalez *et al.*, 1994). To be able to sense these changes, the CBs are the most perfused organ per gram weight in the body (2000 mL/min per 100 mg of tissue) and receive blood via an arterial

branch arising from internal or external carotid artery (Gonzalez *et al.*, 1994; Paton *et al.*, 2013). Also, in order to respond to these changes , the CBs are innervated by the nerve fibers from the glossopharyngeal (carotid sinus nerve, CSN), vagal, and the sympathetic nerve of the nearby superior cervical ganglion (González *et al.*, 1995; Kumar and Prabhakar, 2007; Paton *et al.*, 2013).

The CB is organized into clusters of cells, glomeruli, which are in close proximity with a vast network of capillaries and connective tissue. The glomerulus are constituted mainly by two types of cells: the chemoreceptor cells, also known as glomus or type I cells, which are derived of the neural crest and are synaptically connected with the CB' sensitive nerve, the CSN (Gonzalez *et al.*, 1994) and, the type II cells or sustentacular cells. Type II cells formerly believed that they only had a supportive role, but recently has been proposed that they are adult neural stem cells that in response to a stimuli, like hypoxia, can proliferate and differentiate into new type I cells (Conde *et al.*, 2014; Iturriaga and Alcayaga, 2004; Pardal *et al.*, 2007; Piskuric and Nurse, 2013).

The stimulation of the CBs leads to the release of neurotransmitters (as CA, adenosine, adenosine triphosphate (ATP), serotonin, acetylcholine, neuropeptides) that lead to an increase in the action potential frequency of the CSN (Conde *et al.*, 2014). The CSN activity is integrated in the brainstem to induce a panoply of respiratory reflexes in order to normalize blood gases via hyperventilation and to regulate blood pressure (BP) and cardiac performance via activation of the sympathetic nervous system (Gonzalez *et al.*, 1994; Marshall, 1994).

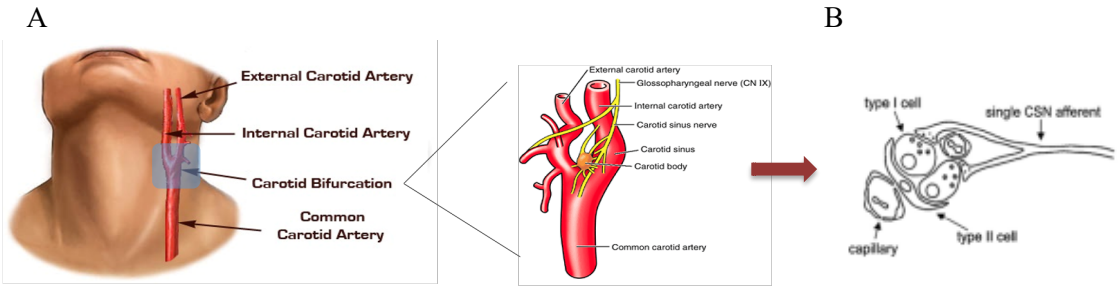

Figure 1. 8. Carotid body (CB) location and innervation (A), with the celular arragenment (B). (adapted from Gríofa *et al.*, 2014).

1.4.1. Carotid Body, Metabolic Diseases and the Sympathetic Nervous System

It is consensual that metabolic diseases are associated with sympathetic overactivation (Esler *et al.*, 2006; Lambert *et al.*, 2010; Paton *et al.*, 2013; Ribeiro *et al.*, 2013; Tentolouris, Liatis and Katsilambros, 2006) . Also, an overactivation of the SNS is related with insulin resistance, but the link between these defects it is not yet fully understood (Egan, 2003; Iturriaga *et al.*, 2016; Tentolouris, Liatis and Katsilambros, 2006; Tsioufis *et al.*, 2007, 2011). Prof. Conde group has established that the CB is the link between sympathetic nerve activity and insulin resistance (Ribeiro *et al.*, 2013, Sacramento et al. 2017), as they showed that resection of CSN prevented and normalized the overactivation of the SNS induced by hypercaloric diets, measured as plasma and adrenal medulla catecholamines and by the measurement of heart rate variability (Sacramento *et al.*, 2017).

In the last years, a rift of opinions has emerged regarding the effect of hypoglycemia (low blood glucose) on the CB. While some authors suggested that hypoglycemia is capable of activating the CB (Pardal and López-Barneo, 2002; Zhang, Buttigieg and Nurse, 2007), others suggested that hypoglycemia per se is not able to activate the CB, as it is incapable of altering CSN frequency of discharges in vivo and ex vivo (Bin-Jaliah, Maskell and Kumar, 2004; Conde *et al.*, 2014; Gallego-Martin *et al.*, 2012). Additionally, it was also observed that hyperglycemia (25mM of glucose) did not changed either the basal CSN activity or the CSN chemosensory activity in response to hypoxia (0% O_2), suggesting that hyperglycemia does not activate the CB nor potentiates the response to hypoxia (Conde, Sacramento and Guarino, 2018).

Recently, it was demonstrated that insulin stimulates the peripheral chemoreceptors located in the CB, suggesting that probably hyperinsulinemia might be a key factor initiating CB overactivation, leading to the increased SNS activity (Ribeiro *et al.*, 2013). Insulin is capable of stimulating the CB and inducing a neurosecretory response by binding to the insulin receptor present in the chemoreceptor cells of the CB (Ribeiro *et al.*, 2013). Insulin induces an increase in intracellular Ca^{2+} in the chemoreceptor cells, stimulating the release of ATP and dopamine from the CB (Ribeiro *et al.*, 2013). Also, insulin produces a dose-dependent increase in ventilation, an effect that is abolished by

CSN cut (Conde *et al.*, 2014; Guarino *et al.*, 2013). Therefore, these results are in agreement with the hypothesis that insulin is a stimulus for CB activation, independently of glucose levels. Shifting the paradigm of CB activation from "low glucose" to "high insulin".

Moreover, an overactivation of the CB can be perceived as a dysfunction of the organ, since, evidence suggests that CB are overactive in metabolic diseases like T2D, hypertension and obesity (Conde, Sacramento and Guarino, 2018; Sacramento *et al.*, 2017). The CB activity lead to an increase in the action potential frequency of the CSN, so an overactivation of these organ leads to overstimulation of the CSN (Conde *et al.*, 2014; Conde, Sacramento and Guarino, 2018). The CB sympatho-stimulation though the CSN, leads to an activation of the SNS (Sacramento *et al.*, 2017). The CB overactivation has been demonstrated to be connected with the development of insulin resistance and glucose intolerance (Conde, Sacramento and Guarino, 2018; Ribeiro *et al.*, 2013; Sacramento *et al.*, 2018). Conde's lab demonstrated, that by abolishing CB activity, with the denervation or the bioelectronic modulation of the CSN was possible to prevent and reverse insulin resistance (Figure 1.9.), as well as glucose intolerance (Table 1.3) in young animals with prediabetes and in an early stage of T2D (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017, 2018).

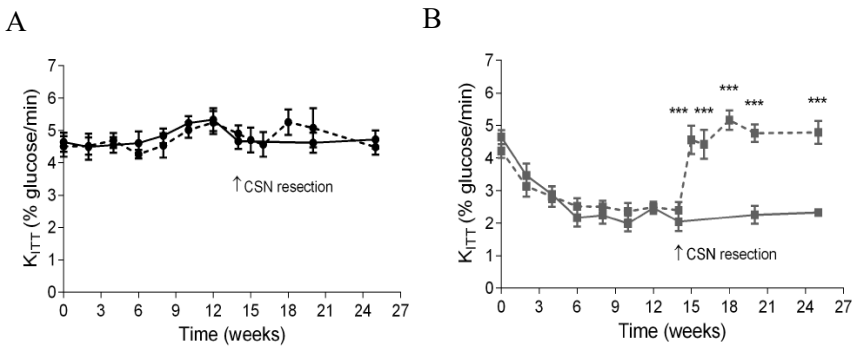

Figure 1. 9. Effect of the carotid sinus nerve (CSN) resection on the insulin sensitivity. Effect of CSN resection on insulin sensitivity assessed by an insulin tolerance test (ITT) and expressed as the constant rate for glucose disappearance (K_{ITT}) in young controls (CTL) (A) and HFHSu (B) animals. CSN resection was performed at 14 weeks of diet and animals were maintained under their respective diets until the 25 weeks. Black line, control sham; Black dotted line, control with CSN resection; Grey line, HFHSu sham; Grey dotted line, HFHSu with CSN resection. Data are means ± SEM of 8-10 animals. One and Two-Way ANOVA with Dunnet's and Bonferroni multicomparison test, ***p<0.001 vs without CSN resection (Sacramento *et al.*, 2018).

Table 1.3. Area under the curve (AUC) obtained from the analysis of the glucose excursion curves in young control (CTL) and high-fat high-sucrose (HFHSu) animals with or without carotid sinus nerve (CSN) resection (Sacramento *et al.*, 2018)**.**

Area under curve (mmol/l x min)	Baseline	25 weeks of diet
CTL Sham	1254 ± 27	1192 ± 25
CTL – CSN resection	1276 ± 17	1141 ± 58
HFHSu Sham	1256 ± 38	1372 ± 31**
HFHSu – CSN resection	1252 ± 29	1263 ± 22†

Data are means ± SEM of 8-10 animals. One and Two-Way ANOVA with Dunnet's and Bonferroni multicomparison test: ***p*<0.01, vs control; †*p*<0.05, with vs without CSN resection.

Conde's lab not only showed that the modulation of the CSN prevents and reverses insulin resistance and glucose intolerance, but also that improves plasma fasting glucose (Table 1.4.) and decreases perinephric fat accumulation (Table 1.5.) (Sacramento *et al.*, 2018).

Table 1.4. Effect of carotid sinus nerve resection (CSN) on plasma fasting glucose (mg/dl) in control (CTL) and HFHSu young animals (Sacramento *et al.*, 2018).

Treatments	Models	Baseline	25 weeks of diet
Blood glucose (mmol/l)	CTL Sham	5.08 ± 0.13	4.81 ± 0.13
	CTL – CSN resection	5.07 ± 0.10	4.74 ± 0.30
	HFHSu Sham	4.80 ± 0.15	5.47 ± 0.19***
	HFHSu – CSN resection	4.80 ± 0.19	4.90 ± 0.16†,ǂ

Data are means ± SEM of 8-10 animals. One and Two-Way ANOVA with Dunnet's and Bonferroni multicomparison test, ****p*<0.001 vs control; †*p*<0.05 vs without CSN resection; ǂ*p*<0.05 vs without CSN resection at 25 weeks of diet.

Table 1.5. Effect of carotid sinus nerve (CSN) resection on total, perinephric, perienteric and epididymal fat in control and HFHSu young animals (Sacramento *et al.*, 2018).

	CTL Sham	CTL – CSN resection	HFHSu Sham	HFHSu – CSN resection
Total fat (g/kg)	66.44 ± 3.93	64.14 ± 5.16	113.9 ± 9.73***	104.2 ± 7.64
Perienteric fat (g/kg)	15.58 ± 0.82	14.42 ± 1.37	24.44 ± 2.80**	22.97 ± 1.74
Perinephric fat (g/kg)	25.78 ± 1.75	26.03 ± 1.74	52.57 ± 4.45***	44.02 ± 3.22†
Epididymal fat (g/kg)	25.08 ± 1.52	23.68 ± 2.77	36.90 ± 2.83**	37.17 ± 3.19

Data are means ± SEM of 8/10 animals. One and Two-Way ANOVA with Dunnet's and Bonferroni multicomparison test, **p<0.01, ***p<0.001 vs control; †p<0.05 vs without CSN resection.

These results demonstrated that the overactivation of the CB is in the genesis of insulin resistance and of the decreased of peripheral insulin action seen in metabolic disorders, like T2D, prediabetes and MS (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017). Also, these results highlight that the link between the CB and the SNS must be further investigated to better understand its role on the control of glucose metabolism, insulin resistance and metabolic disturbances. They also highlight that the modulation of the CB activity might arise as a new therapeutic approach for the treatment of sympathetically mediated diseases.

1.5. Aging

Aging is described as a series of morphological and functional changes over time (Bonomini, Rodella and Rezzani, 2015; Guarner and Rubio-Ruiz, 2011). It is seen as a biological process characterized by a progressive deterioration in physiological functions, with a decline in maintenance of homeostasis, accompanied by an array of metabolic changes, including insulin resistance, changes in body composition and modulation of mitochondrial function (Barzilai *et al.*, 2012; Bonomini, Rodella and Rezzani, 2015; Finkel, 2015). These changes contribute to an increased predisposition to age-related conditions, like T2D, hypertension, and CVD (Bonomini, Rodella and Rezzani, 2015; Finkel, 2015; Riera and Dillin, 2015). Also, several disorders whose prevalence increase with aging, such as obesity, insulin resistance, inflammation,

changes in the activity of the hypothalamus-hypophysis suprarenal axis, stress and hypertension, also contribute to an increase of the metabolic diseases prevalence (Bonomini, Rodella and Rezzani, 2015; Guarner-Lans *et al.*, 2011; Guarner and Rubio-Ruiz, 2011).

Insulin resistance, a major component of MS and T2D, is commonly observed in older people (Barzilai *et al.*, 2012; Morley, 2008). It is described in humans and animal studies that aging is associated with a progressive decline in insulin action and a decrease in insulin sensitivity (Barzilai *et al.*, 2012; Escrivá *et al.*, 2007; Guarino *et al.*, 2013; Morley, 2008; Santulli *et al.*, 2012). A possible hypothesis for the association between aging and insulin resistance comprehends four main pathways: environmental causes, mainly diet styles and physical activity; anthropometric changes, it is well known that aging is associated with a relative increase in body fat and a decrease in lean body mass, muscle tissue especially, which leads to a reduction in active metabolic tissue; neuro-hormonal variations, with age comes a decline in plasma insulin-like growth factor-1 (IGF-1) and dehydroepandrosterone sulphate (DHEAS), which might have an effect opposite to that of insulin at the skeletal muscle and adipose tissue levels; and finally the rise in oxidative stress with age (Barbieri *et al.*, 2001). Additionally, a decrease in glucose tolerance with age has been recognized since 1920 (DeFronzo, 1981). Results obtained in older subjects submitted to an oral glucose tolerance test demonstrated that the ability to metabolize glucose is impaired in these subjects (DeFronzo, 1981). Therefore, aging is associated to a progressive decline in glucose tolerance, triggered by a tissue desensitization to insulin (Barzilai *et al.*, 2012; DeFronzo, 1981).

The aging process is altered or accelerated in metabolic and cardiovascular diseases. MS is accompanied with vast systemic complications but it is also associated with an early aging (Bonomini, Rodella and Rezzani, 2015; Guarner-Lans *et al.*, 2011; Guarner and Rubio-Ruiz, 2011; Nunn, Bell and Guy, 2009). Although, aging and the development of insulin resistance are seem to be accelerated in the MS, the mechanisms behind the precocious aging in MS remain elusive (Fadini *et al.*, 2011; Guarner-Lans *et al.*, 2011; Guarner and Rubio-Ruiz, 2011).

Abdominal obesity, commonly observed with aging, is a major contributor to insulin resistance and MS (Barzilai *et al.*, 2012; Folsom *et al.*, 1993). Moreover, aging is associated with an increase in bodyweight, especially in subcutaneous and visceral body fat and studies have shown that when obesity is present there is an upregulation of the aging pathways (Catalano, Bergman and Ader, 2005; Guarner and Rubio-Ruiz, 2011). Additionally, individuals with obesity have a greater risk for the development of T2D, CVD, hypertension and hypercholesterolemia, and vice versa (Ryan, 2000).

Aging is also associated with an increase in proinflammatory cytokines, which are known to interfere with insulin action (Barzilai *et al.*, 2012). This increase results from the age-associated accumulation of visceral fat but also from the increasing numbers of senescent cells (Barzilai *et al.*, 2012; Sepe *et al.*, 2011). Hence, inflammation is a key factor in the progressive loss of lean tissue and impaired immune function observed in aging. An elderly immune system becomes highly susceptible to chronic inflammatory reactions and is less able to respond to an acute event of new antigens (Guarner and Rubio-Ruiz, 2011; Martinis *et al.*, 2005; Wu *et al.*, 2007). There are several predisposing factors for the chronic inflammation that occurs during aging, including, increased oxidative stress and an increased incidence of asymptomatic bacteriuria (Martinis *et al.*, 2005). In addition, to produce more inflammatory cytokines, adipocytes from old mice induce a higher inflammatory response in other cells (Wu *et al.*, 2007).

These age-related alterations in metabolism and body fat distribution are active participants in a vicious cycle that can accelerate the aging process and the onset of MS, insulin resistance and cardiovascular diseases, making MS of predominant importance as a growing epidemic in a worldwide aged population (Barzilai *et al.*, 2012; Bonomini, Rodella and Rezzani, 2015; Haffner and Taegtmeyer, 2003).

1.5.1. Aging and Carotid Body

The CB might play an important role during aging, as this organ has the ability to influence body homeostasis (Barzilai *et al.*, 2012; Bonomini, Rodella and Rezzani, 2015; Finkel, 2015; Giulio *et al.*, 1974). Ageing also increases the predisposition to age-related conditions, like T2D, hypertension, and CVD (Bonomini, Rodella and Rezzani, 2015; Finkel, 2015; Riera and Dillin, 2015). Knowing that the deregulation of

the CB is linked to an overactivation of the SNS and to metabolic diseases (Conde, Sacramento and Guarino, 2018; Sacramento *et al.*, 2017), the CB might have a critical role in age-induced metabolic dysfunctions.

The aging CB shows an attenuated response to hypoxia and suffers age-dependent structure modifications (Giulio *et al.*, 1974; Giulio, Di *et al.*, 2003). Ultimately, aging leads to CB adaptation through a reduction to the sensitivity to stimuli (Giulio *et al.*, 1974; Giulio, Di *et al.*, 2003). So the overall basal oxygen reduction of body requirements may be a mechanism of cellular self-protection to the accumulation of reactive oxygen species (ROS) during aging (Giulio *et al.*, 1974; Giulio, Di *et al.*, 2003). Moreover, aging leads to a reduction in synaptic connections between chemoreceptor cells, as well as a reduction of nerve conductivity (Finkel and Holbrook, 2000; Giulio *et al.*, 1974, 2012; Giulio, Di *et al.*, 2003; Rivner, Swift and Malik, 2001). Also, the maximum number of impulses per minute in nerves generally decrease and so does sensitivity by peripheral receptors, leading to a reduction of the homeostatic capacity with higher latency to stimuli (Finkel and Holbrook, 2000; Giulio *et al.*, 1974, 2012; Giulio, Di *et al.*, 2003; Rivner, Swift and Malik, 2001).

The aged CB also shows a decrease in the number of cells associated with an increase in extracellular matrix, a reduction in number and volume of type I cells as well as in mitochondria (Dymecka, Walsky and Pokorski, 2006; Giulio *et al.*, 1974; Giulio, Di *et al.*, 2003; Sacramento *et al.*, 2019). Pokorski *et al*. showed advanced degenerative morphological changes in the CB's parenchyma, affecting predominantly the type I cell, the ones with the chemoreceptor capacity, whereas type II CB cells remain unaltered (Giulio *et al.*, 1974; Pokorski *et al.*, 2004). These factors suggest that the CB becomes less responsive, which could explain the observed decrease in chemosensory responses during aging (Giulio *et al.*, 1974). Also, Conde *et al.* demonstrated that in spite of an enlargement of the organ, there is an age-dependent decrease in parenchymatous tissue evident after 12 months of age (Conde *et al.*, 2006). The enlarged CB showed a higher CA content and turnover time and reduced responsiveness to hypoxic stimuli (Conde *et al.*, 2006; Sacramento *et al.*, 2019).

Recently, it has been shown in young animals with prediabetes and in an early stage of T2D that the abolition of the CB activity through the CSN resection or the bioelectronic modulation of CSN activity prevented and restored the insulin sensitivity and glucose tolerance (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017, 2018). With the social burden of metabolic diseases and the increasing prospective with age it is imperative to explore the abolishment of CB activity in older rats as a potential therapeutic intervention in metabolic diseases. Therefore, further studies are required to fully clarify the role and value of the CB activity in age-induced metabolic dysfunctions, as this organ suffers its own modifications with age.

2. Aims

The metabolic diseases are a major public-health epidemic with increasing incidence and prevalence worldwide. Aging itself leads to a greater predisposition for these conditions, like T2D, NAFLD hypertension, CVD, obesity and insulin resistance. Therefore, it is of significant importance to find new therapeutic targets for these diseases in an aged world population. Knowing that an overactivation of CB activity and consequent increase in the SNS activity is associated with the development of insulin resistance and glucose intolerance in young rats, and that the CSN resection prevents and reverts these metabolic features, the **general aim** of this thesis was to investigate the impact of the abolishment of CB activity in metabolic function in older rats as the modulation of CSN activity might be a potential therapeutic intervention in metabolic diseases, like T2D, dyslipidemia and NAFLD in aging.

The **specific aims** of this thesis are:

- To evaluate the impact of long-term hypercaloric diets consumption on glucose homeostasis in old animals.
- To study the effect of the CSN resection on age-induced metabolic dysfunction in 15 months old rats.
- To investigate the effect of long-term hypercaloric consumption on liver function in young and old animals.
- To evaluate the effect of the CSN resection on liver function in control and long-term hypercaloric consumption young and old rats.

3. Methods

3.1. Animal Model and Experimental Procedure

Experiments were performed in male Wistar rats, ageing 8-10 weeks old, acquired from the NOVA Medical School|Faculdade de Ciências Médicas, Universidade Nova de Lisboa animal house. The animals were divided randomly into two groups (Figure 3.1.): two control (CTL) groups that were fed with a standard diet (7.4% fat + 75% carbohydrate (4% sugar) + 17% protein, Probiológica, Sintra, Portugal) and two high-fat high-sucrose (HFHSu) groups that were fed with a lipid-rich diet (60% energy from fat): 34.9% fat + 25.9% carbohydrate + 23.1% protein; TestDiet, St. Louis, USA) plus sucrose (35%) in drinking water, during 14 and 44 weeks.

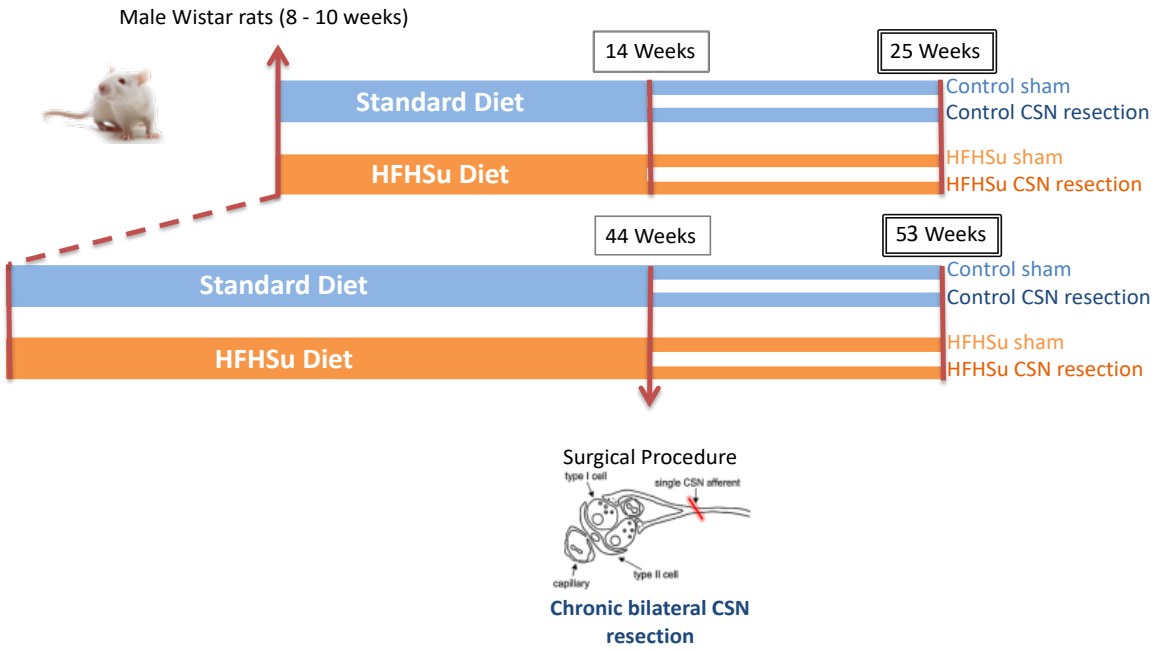

Figure 3. 1. Representative scheme of the experimental protocol performed on Wistar rats of 25 and 53 weeks and their respective diets.

After the respective diet periods the animals were randomly divided and half of the group was submitted to a surgical procedure to chronically resect the CSN and the other half to a sham surgical procedure, in which the animals were submitted to the same protocol but in which the nerves remained intact.

The surgical protocol includes: intraperitoneal anesthesia with medetomidine (0.5 mg/kg) (Sedator©, Siloal, Coimbra, Portugal) and ketamine (75 mg/kg) (Nimatek©, Dechra, Northwich, United Kingdom) and its reversion with antipamezole (0.25mg/kg, i.p.) (Antisedan©, Esteve, Lisbon, Portugal). After surgery, when the animals were awake an analgesic, buprenorphine (10 mg/kg, s.c.) (Bupaq©, Richter pharma, Budapest, Hungary), was administered. Additionally, rats were treated post-operatively for 2-3 days with an anti-inflammatory drug carprofen (5 mg/kg, s.c.) (Rimadyl©, Zoetis, New Jersey, USA).

Animals were maintained on their respective diets after the surgery during nine weeks in old animals and 11 weeks in young animals. The difference in post-surgical periods is due to the regeneration of the CSN. The study in young animals was performed previously (results already published in Sacramento *et al.* 2018) and the rats were maintained for 11 weeks after the surgery. However, the CSN starts to regrow 6 days after being cut and usually it completely regenerates after 11–12 weeks (Zapata, Stensaas and Eyzaguirre, 1976). Therefore, in the following studies the post-surgical period was altered to 9 weeks, the time used in the present study in aged animals. At the end of this period, which corresponds to 25 and 53 weeks on diet, the animals were submitted to a terminal experiment, in which animals were anesthetized with pentobarbital sodium (60 mg/kg i.p.) (Eutasil©, Ceva, Libourne, France) and blood was collected by heart puncture and tissues such as the liver, soleus and gastrocnemius, diaphragm and visceral, perinephric, epididymal and brown adipose tissues (BAT) (collected from the interscapular region), brain and hypothalamus samples were rapidly collected and stored at -80 ºC until posterior analysis. A smaller portion of the liver, BAT and white adipose tissue (WAT) were immediately set aside and preserved in formalin (4ºC). Plasma was collected to ethylenediaminetetraacetic acid (EDTA) precoated tubes, and kept on ice. After, the plasma samples were centrifuged (Sigma, Madrid, Spain) at 3,000 × *g* for 10 min (4 °C) and stored at −80 °C in an ultralow freezer (Guarino *et al.*, 2013).

Caloric and liquid intake, body weight and animal behavioral changes were assessed once a week, before and after the surgical procedure in all groups of animals. Additionally, insulin sensitivity and glucose tolerance were evaluated throughout the

experimental protocols by an insulin tolerance test (ITT) and an oral glucose tolerance test (OGTT), respectively.

Principles of laboratory care were followed in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU). Experimental protocols were approved by the Ethics Committee of the NOVA Medical School.

3.2. Metabolic Evaluation

3.2.1. Measurement of the Insulin Sensibility

The ITT provides an estimation of the insulin sensitivity *in vivo*. For the ITT, the animals were fasted overnight with free access to water. In the morning, the basal glycemic levels were measured and afterwards a bolus of insulin (0.1 U/kg, Humulin® R 100IU/ml, Lilly) was administrated in the tail vein of the animals (Monzillo *et al.*, 2003; Sacramento *et al.*, 2015). Glycemia levels were then measured every minute during 15 minutes and the decrease of the plasmatic glucose concentration examined. The blood was collected by tail tipping and evaluated using a glucose meter (Precision Xtra Meter, Abbot Diabetes Care, Portugal) and test strips (Abbot Diabetes Care, Portugal). The constant rate for glucose disappearance (K_{ITT}) was used to evaluate the insulin sensitivity, knowing that when the K_{ITT} is > 3.5 means that the animals are insulin sensitive and when the K_{ITT} is < 3.5 the animals are insensitive to insulin action, meaning that the animals are insulin resistant. The constant is obtained from the equation:

$$K_{ITT} = \frac{0.693}{t_{\frac{1}{2}}} \times 100$$

In which the value of the glucose half-time ($t_{\frac{1}{2}}$) represents the mean lifetime of the glucose and was calculated from the slope of the curve during the linear phase of the plasma glucose disappearance by the analysis of the least squares method (Monzillo *et al.*, 2003; Sacramento *et al.*, 2015).

3.2.2. Measurement of Glucose Tolerance

Animal glucose tolerance was evaluated through an OGTT. The OGTT allowed to determine the glucose tolerance and subsequently the speed of release of insulin and its action in the peripheral tissues (Monzillo *et al.*, 2003).

The OGTT consisted in the administration of a glucose solution (2 g/kg) by gavage to overnight fasted animals. The blood glucose levels were measured by tail tipping before the glucose administration and at 15, 30, 60, 120 and 180 minutes after the gavage (Kinzig, Honors and Hargrave, 2010). The glucose tolerance was evaluated as the area under the curve (AUC) of the glucose excursion curves.

3.3. Lipid profile Evaluation

Lipid profile was determined using the plasma collected from the animals previously centrifuged and a RANDOX kit (RANDOX, Porto, Portugal), where the total cholesterol and TG were measured by a trinder-based colorimetric end-point assays (Sacramento *et al.*, 2017).

3.4. Liver´s Lipid Quantification

3.4.1. Liver´s Total Lipid Content

In order to evaluate and compare the total liver lipid content a modified protocol described by Folch was used (Folch, Lees and Sloane Stanley, 1957). Briefly, a portion of the liver was homogenized (Tube A) using an automatic homogenizer (IKA, Germany) in Folch' solution (Chloroform: Methanol 2: 1 v/v).

The homogenization of the tissue was always performed with the following proportions: 3 ml of Folch' solution per 0.5 g of liver.

Afterwards, the samples were shaken for 2 hours in an automatic shaker and filtered with filter paper into another glass test tube (Tube B). Then, to the tissue remaining in the tube A 2.5 ml of the Folch' solution was added, and the mixture was stirred again for 2 hours, and then filtered. This procedure was repeated twice (Figure 3.2.), always collecting the filtrate into the tube B.

Subsequently, to the filtrates collected in the tube B was added 2 ml of 0.73% NaCl, after which the tube was stirred vigorously and left to rest overnight. After the resting period, was possible to observe two phases in the tube B, the organic and aqueous phase. On the top was located the aqueous phase, predominantly composed of water and methanol, whereas the lower or organic phase, mainly composed of chloroform, possesses the capacity to retain the lipids. (Liquid Extraction: Folch). Therefore, the phase located at the bottom of the tube B was collected to a previously weight tared eppendorf.

Furthermore, to the phase remaining in the tube B it was added a solution of Folch's reagent: NaCl 0.53% (80:20), which was stirred and left to rest for 2 hours. Afterwards, the organic phase was collected to the same eppendorf in order for it to dry. Finally, the eppendorfs were weighted again and the percentage of lipids in the tissue was determined, according to the initial liver´s weight used in the experiment.

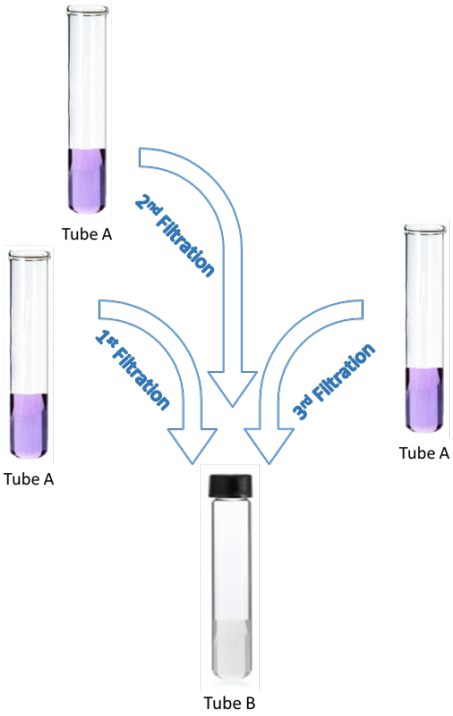

Figure 3. 2. Scheme from the Folch' adapted protocol. The liver homogenates contained in tube A were shaken for 2 hours and filtered into glass test tube B. To the tissue remaining in the tube A 2.5 ml of the Folch' solution was added, and the mixture was stirred again for 2 hours, and then filtered. This procedure was repeated twice, always collecting the filtrate into the

3.4.2. Liver´s Triglyceride Content

The triglyceride content of the livers was evaluated using the final dried samples of the analysis of the liver's total lipid content with the Triglyceride Assay Kit-Bulk 500 Point Assay Kit (ZENBIO, NC, USA).

3.5. Histologic Analysis

After collecting and preserving the tissues in formalin for at least 72 hours at 4ºC the tissues were sent to the histology facility at CEDOC. The livers were divided into two sections: half for paraffin embedding tissue sections and the other half to be crio preserved. The crio preserved samples were embedded in optimal cutting temperature (OCT) compound and snap frozen in liquid nitrogen and kept at -80ºC for posterior analysis.

For the preparation of tissue sections for hematoxylin & eosin (H&E) staining, the tissues were embedded in blocks of paraffin. For the treatment of the samples and the paraffin processing the Automated Leica Tissue Processor© (TP1020) was used. The samples are placed in cassettes and then emerged in the cylindric canisters of the Automated Leica Tissue Processor©. The samples stayed 1:30 hours in each cannister and the same solution was put into two subsequent cannisters. The first canister is filled with alcohol 70% to dehydrate the samples, next they are emerged into a new solution of alcohol 70%. After the samples were submerged into alcohol 96% and then again into the a new cannister with the same solution. Next, following the same procedure the samples were put into alcohol 100%, xylene (klinipath, Gelderland, Nederland) and to finish into paraffin at 70ºC (Diapath, Bergamo, Italy). The liver samples were cut in 3 µm thickness in the microtome (Microm HM 200) and placed in the slides.

For the H&E staining the slides containing the sample were deparaffinized in xylene for 15 minutes and hydrated by been immersed for 30 seconds in each alcohol (100%, 96% and 70%) and finally, in distilled water for 5 minutes. The slides were emerged in Harris Hematoxylin (Merck, New Jersey, USA) for 10 minutes, then dipped in distilled water to remove the excessive stain. After, they were differentiated in Acid Alcohol 1% with 3 dips (1 second each) and were washed in running tap water for 5 minutes. Then, the slides were dipped in Alcohol 70% and four times (1 second each) in Alcoholic Eosin Y (Sigma-Aldrich, Missouri, USA). The slides were dehydrate in alcohol (70%, 96%, 100%) 30 seconds each and cleared in xylene. Finally, the slides were left to set for at least 10 minutes before they were sealed with the lamella. The H&E staining marked the nuclei with blue and the cytoplasm with pink. The slides and paraffin blocks were kept at room temperature.

After liver H&E slides preparation they were sent to Instituto de Medicina Molecular (IMM) where they were analyzed for the presence of hepatocellular damage, fibrosis, fatty change and inflammation according with table 3.1.

Table 3.1. Hematoxylin and Eosin (H&E) Liver Scores

Hepatocellular damage scores	Fibrosis scores	Fatty change/ Steatosis scores	Immune cell infiltration scores
1: minimal	1: minimal	1: minimal	1: minimal
2: mild	2: mild	2: mild	2: mild
3: moderate	3: moderate	3: moderate	3: moderate
4: marked	4: marked	4: marked	4: marked
5: extreme	5: extreme	5: extreme	5: extreme

3.6. Data Analysis

The data analysis was assessed with Graph Pad Prism Software version 6 (GraphPad Software Inc., San Diego, CA, USA) and shown as the mean values with their standard errors (Mean ± SEM). The significance of the differences between the mean values was calculated by one- and two-way ANOVA with Bonferroni multiple comparison tests. Differences were considered statistically significant at $p<0.05$.

4. Results

In the present thesis the impact of long-term diet consumption and CSN resection on body weight gain, fasting glucose, insulin sensitivity, glucose tolerance and fat depots were studied in rats fed with a standard or HFHSu diet during 53 weeks was evaluated. However, since this study also focus on the effect of age and long-term hypercaloric consumption in the liver metabolism, samples of liver collected from HFHSu animals submitted to hypercaloric diets during 25 weeks and CSN resection from a previous study were used to analyzed the total liver lipid content, liver TG and cholesterol as well as lipid deposition, inflammation and fibrosis.

4.1. Effect of long-term hypercaloric diet intake and carotid sinus nerve resection on body weight

Figure 4.1. describes the effect long-term hypercaloric intake and the effect of the chronic CSN resection on body weight in aged animals (Figure 4.1 A and B).The standard diet with 2.56 Kcal/g was able to stimulate an increase in CTL rats before the surgery (CTL before sham surgery =1.33±0.07g/day; CTL before CSN denervated =1.21±0.03g/day) (Figure 4.1 B). Body weight decreased by 63.40% with sham surgery in control animals and by 102.63% with CSN resection . Hypercaloric diet, with 6.46 Kcal/g, increased as expected body weight of the animals (HFHSu sham =1.44±0.22g/day; HFHSu CSN denervated =1.52±0.37g/day) (Figure 4.1 B), an effect that was reduced by 47.22% after the sham surgery and 90.21% after the CSN resection .

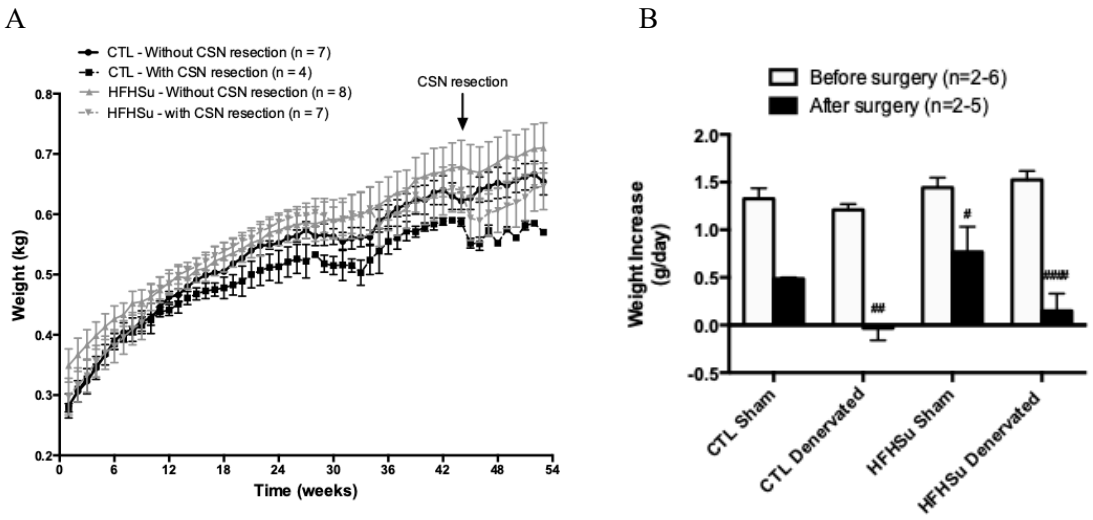

Figure 4. 1. Effect of long-term hypercaloric diet consumption and of carotid sinus nerve (CSN) resection on the body weight (A) and body weight change in old controls (C TL) and HFHSu animals. (A) Black line with dots represent body weight of CTL animals without CSN resection; dotted black line with squares represent body weight of CTL animals with C SN resection; grey line with triangles represent body weight of HFHSu animals without CSN resection; dotted grey line with inverted triangles represent body weight of HFHSu animals with CSN resection. (B) White bars represent the change in body weight before the CSN resection and sham procedure; black bars represent the change in body weight after the CSN resection and sham procedure. Bars represent mean±SEM of 1-6 values. One- and Two-Way ANOVA with Bonferroni multicomparison tests; #p<0.05, ##p<0.01 and ####p<0.0001 comparing within the group .

4.2. Effect of long-term hypercaloric diet intake and carotid sinus nerve resection on fasting glucose in old animals

Table 4.1. shows the effect of long-term hypercaloric diet intake and the chronic CSN resection on plasma fasting glucose in old animals. Previously, Sacramento *et al.* (2017), observed, in young animals, a 13.96% increase in plasma fasting glucose with hypercaloric diet consumption and a reduction of 10.42% with the CSN resection (Sacramento *et al.*, 2017). Herein, in old animals neither long-term hypercaloric diet consumption nor CSN resection had impact in fasting glycemia.

Table 4.1. Effect of carotid sinus nerve resection (CSN) on fasting glucose (mg/dl) control (CTL) and HFHSu animals.

	CTL		HFHSu	
	Without CSN resection	With CSN resection	Without CSN resection	With CSN resection
Before diet	77.00 ± 2.39	76.50 ± 4.50	79.20 ± 2.04	76.00 ± 2.80
44 weeks of diet	87.88 ±3.32	87.33 ± 8.41	86.22 ± 5.03	87.63 ± 3.09
9 weeks after surgery	84.57 ± 6.35	83.75 ± 2.29	87.50 ± 3.96	84.43 ± 4.358

Data are means±SEM of 4-8 values.

4.3. Effect of long-term hypercaloric diet intake and carotid sinus nerve resection on insulin sensitivity in old animals

Figure 4.2. shows the effect of long-term hypercaloric diet intake as well as the effect of the chronic CSN resection on insulin sensitivity in old animals.

As expected, age induced a 59.11% decrease in insulin sensitivity from a control value of 4.50±0.36% glucose/min (Figure 4.2. A). When the effects of age and diet were combined, insulin resistance was not significantly aggravated (K_{ITT} CTL 44 weeks of

diet=1.84±0.11% glucose/min; K_{ITT} HFHSu 44 weeks of diet=1.73±0.18% glucose/min) (Figure 4.2. A and B).

Figure 4.2.C and D shows that effect of the chronic bilateral CSN resection on ageing animals. After the 9 week period of follow-up the control and HFHSu animals submitted to the sham procedure remained insulin resistant (Figure 4.2. A and B), with K_{ITT} values similar to values before the surgery (K_{ITT} CTL Sham 44 weeks of diet=1.84±0.11% glucose/min; K_{ITT} CTL 44 weeks of diet without CSN resection=2.20 ± 0.16%glucose/min; K_{ITT} HFHSu Sham 44 weeks of diet=1.73±0.18 % glucose/min; K_{ITT} HFHSu 44 weeks of diet without CSN resection=2.05 ± 0.24%glucose/min).

It can be clearly seen that CSN denervation restores insulin sensitivity in old animals and in old animals submitted to long-term hypercaloric diet consumption. Furthermore, the restoration was maintained along the 9 weeks follow-up, starting in the first weeks after the surgery (Figure 4.2. C and D). In both groups, at the end of the 9 week follow-up period the restore of the insulin sensitivity was maintained (K_{ITT} CTL before diet= 4.30±0.26% glucose/min; K_{ITT} CTL 44 weeks of diet with 9 week CSN resection=4.02±0.37% glucose/min; K_{ITT} HFHSu before diet=4.38±0.19% glucose/min; K_{ITT} HFHSu 44 weeks of diet with 9 week CSN resection=4.30±0.18% glucose/min) (Figure 4.2. C and D).

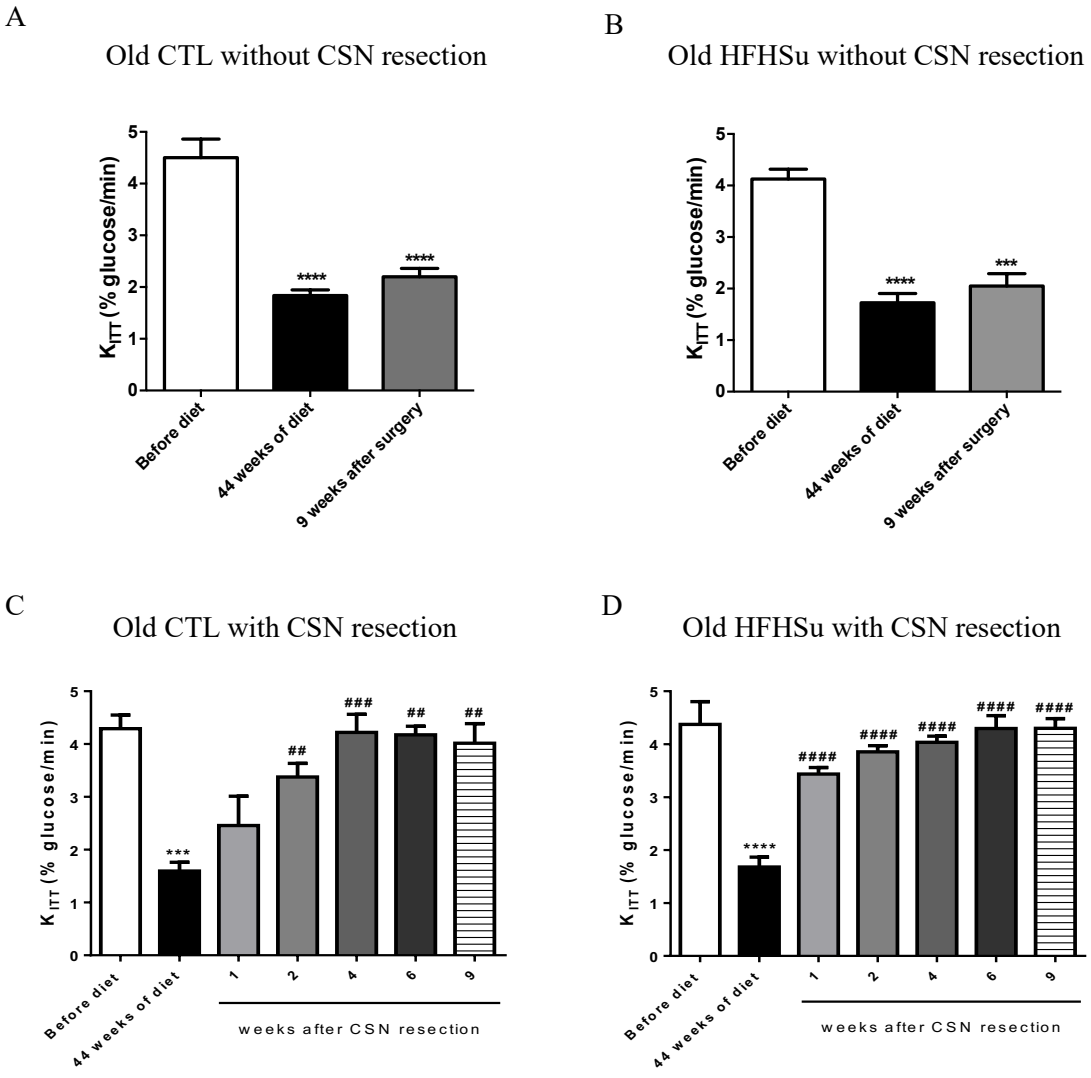

Figure 4. 2. Effect of long-term hypercaloric diet consumption and of carotid sinus nerve (CSN) resection on the insulin sensitivity in old controls (CTL) and HFHSu animals. Control (A) and high-fat high-sucrose (HFHSu) (B) old animals submitted to a sham procedure. C and D) control and HFHSu old animals submitted to the CSN resection, respectively. White bars represent the insulin sensitivity values before the diet, black bars represent the insulin sensitivity values of animals with 44 weeks of their respective diets and grey bars represent the insulin sensitivity values of the weeks after the CSN resection. Bars represent mean±SEM of 4-8 values. One- and Two-Way ANOVA with Bonferroni multicomparison tests; ***p<0.001 and ****p<0.0001 vs control (before diet); ##p<0.01, ###p<0.001 and ####p<0.0001 vs 44 weeks of diet.

4.4. Effect of long-term hypercaloric diet intake and carotid sinus nerve resection on glucose tolerance in old animals

Figure 4.3. and Table 4.2. exhibit the effect of long-term hypercaloric diet intake and the effect of the chronic CSN resection on glucose tolerance.

Age did not modify glucose tolerance (AUC CTL before diet= 21800.75 ±804.43 mg/dl*min; AUC CTL 44 weeks diet=22358.46 ±685.70 mg/dl*min) (Figure 4.3. A, Table 4.2) in rats, however the combination of ageing and diet induced a decrease of 21.19 % in glucose tolerance (Figure 4.3. B. Table 4.2).

CSN resection was shown to improve glucose tolerance, as AUC values obtained from glucose excursion curves showed a decrease of 18.85% and 21.38% with the CSN resection both in CTL and HFHSu old animals, respectively (Figure 4.3. C and D; Table 4.2). CSN resection was able to revert glucose intolerance in the old HFHSu. Moreover, in the animals submitted to the sham procedure it was not observed any significant change in glucose tolerance with the surgery (Figure 4.3. A and B, and Table 4.2.).

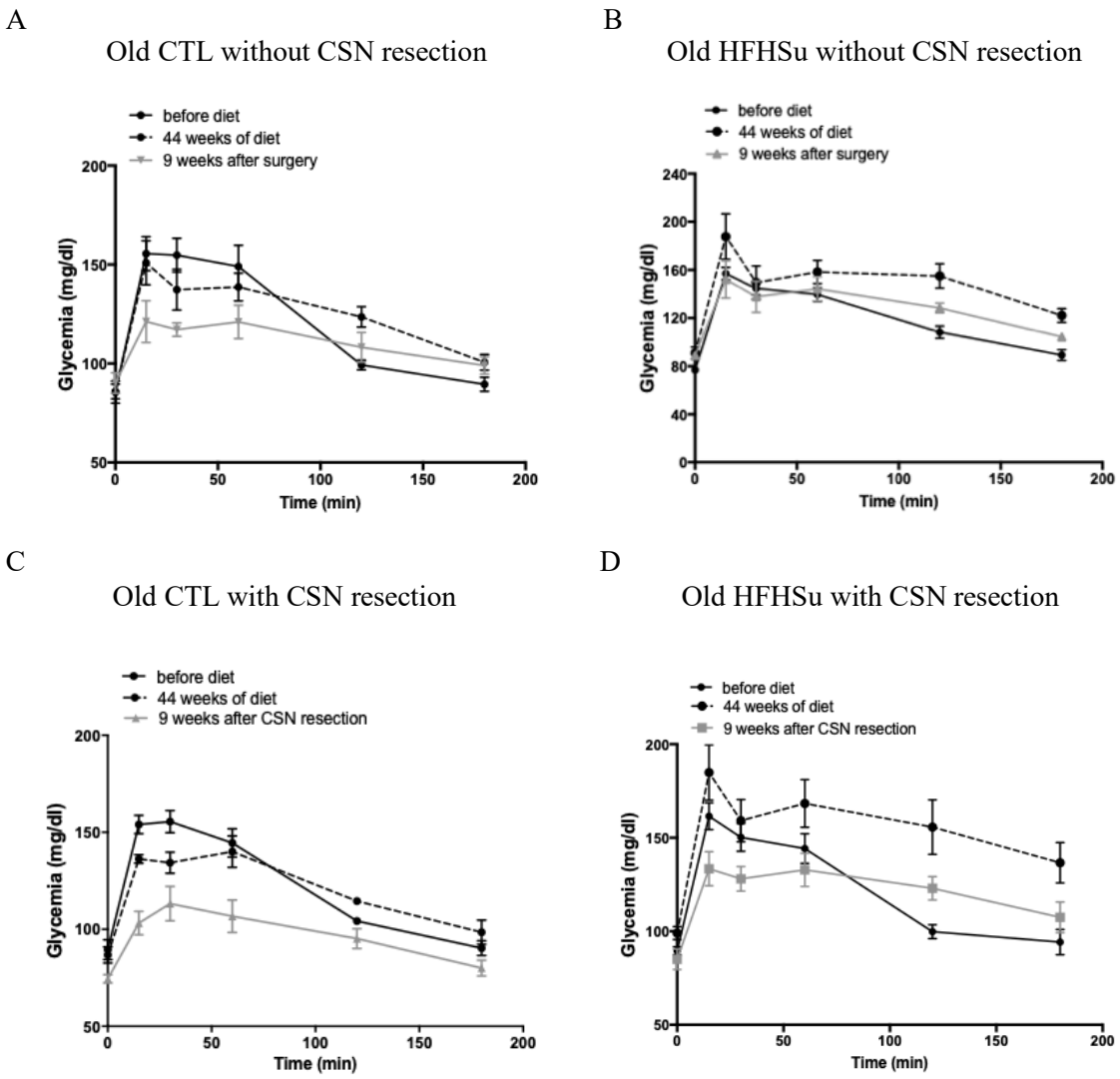

Figure 4. 3. Effect of long-term hypercaloric diet consumption and of carotid sinus nerve (CSN) resection on glucose tolerance in controls (CTL) and high-fat high-sucrose (HFHSu) aged-animals. Control (A) and HFHSu (B) old animals submitted to a sham procedure. C and D) control and HFHSu old animals submitted to the CSN resection, respectively. Black line represents the glucose tolerance values of animals before the diets, black dotted line represent the glucose tolerance values of animals with 44 weeks of diet and the grey line represent the glucose tolerance values of animals after the sham procedure (A and B) and after the CSN resection (C and D). Bars represent mean±SEM of 4-8 values. One- and Two-Way ANOVA with Bonferroni multicomparison tests.

Table 4.2. Effect of long-term hypercaloric diet consumption and of carotid sinus nerve (CSN) resection on the Area under the curve (AUC) obtained from the analysis of the glucose excursion in controls (CTL) and high-fat high-sucrose (HFHSu) aged-animals.

	CTL		HFHSu	
	Without CSN resection	With CSN resection	Without CSN resection	With CSN resection
Before Diet	21800 ± 804	21945 ± 618	21624 ± 543	21778 ± 695
44 weeks of diet	22671 ± 974	21655 ± 429	26953 ± 1705*	28111 ± 2035*
9 weeks post-surgery	20039 ± 1012	17574 ± 1003**	25845 ± 1128	22101 ± 1123#

Data are means±SEM of 4-8 values. Two-Way ANOVA with Bonferroni multicomparison tests; *p<0.05 and **p<0.01 *vs* before diet and #p<0.05 *vs* 44 weeks of diet.

4.5. Effect of long-term hypercaloric diet intake and carotid sinus nerve resection on fat deposition in old animals

In Table 4.3. are depicted the values of the weight of the different fat depots collected from animals after a terminal experimental procedure. Long-term hypercaloric diet consumption increased the amount of total fat by 69.70%, 115.07% the perinephric fat, 50.15% the perienteric fat and by 39.38% the epididymal fat. CSN resection did not modify the fat content in both control and HFHSu old animals.

Table 4.3. Effect of long-term hypercaloric diet consumption and of carotid sinus nerve (CSN) resection on total, perinephric, perienteric and epididymal fat in control and HFHSu old animals.

	CTL		HFHSu	
	Without CSN resection	With CSN resection	Without CSN resection	With CSN resection
Total fat (g/kg)	83.08 ± 14.20	75.27 ± 8.18	140.99 ± 7.43**	138.69 ± 7.31
Perinephric fat (g/kg)	32.84 ± 5.91	28.79 ± 4.12	70.63 ± 6.71***	67.64 ± 5.91
Perienteric fat (g/kg)	19.82 ± 3.58	19.14 ± 1.24	29.76 ± 1.98*	29.82 ± 2.45
Epididymal fat (g/kg)	29.13 ± 4.01	27.34 ± 3.95	40.60 ± 2.82*	41.23 ± 0.95

Data are means±SEM of 6-8 values. Two-Way ANOVA with Bonferroni multicomparison tests; *p<0.05, **p<0.01 and *** p<0.001 *vs* control without CSN resection.

4.6. Effect of long-term hypercaloric diet intake and carotid sinus nerve resection on total cholesterol and triglycerides in young and old animals

Figure 4.4. describes the effect of age, long-term hypercaloric intake and the effect of the chronic CSN resection on lipid profile. Ageing seems to increase total cholesterol levels although non-statistical differences were obtained (p =0,1520). However, when ageing was combined with the long-term consumption of a hypercaloric diet it increases the levels of total cholesterol by 35.39%. CSN denervation had no impact on total cholesterol levels in young or old animals, but in old animals submitted to hypercaloric diets CSN denervation decreases cholesterol levels by 26.40% (Figure 4.4. A). Ageing induced a non-significant statistical increase of 78.92% in TG (CTL 25 weeks = 79.73 ± 12.58 mg/dl; CTL 53 weeks = 142.65±32.35 mg/dl). Hypercaloric diet consumption increased by 69.82% plasma TG in young animals (HFHSu 25 weeks=134.40 ±32.35 mg/dl) and by 49.09% in old animals (HFHSu 53 weeks=118.87±19.33 mg/dl). CSN

resection restored or even lowered below control levels, plasma TG in all groups of animals (TG HFHSu 25 weeks with CSN resection =79.18±29.35 mg/dl, TG CTL 53 weeks with CSN resection =53.80±0 mg/dl; TG HFHSu 53 weeks with CSN resection=40.26 ±9.56 mg/dl), without affecting control young animals (Figure 4.4. B).

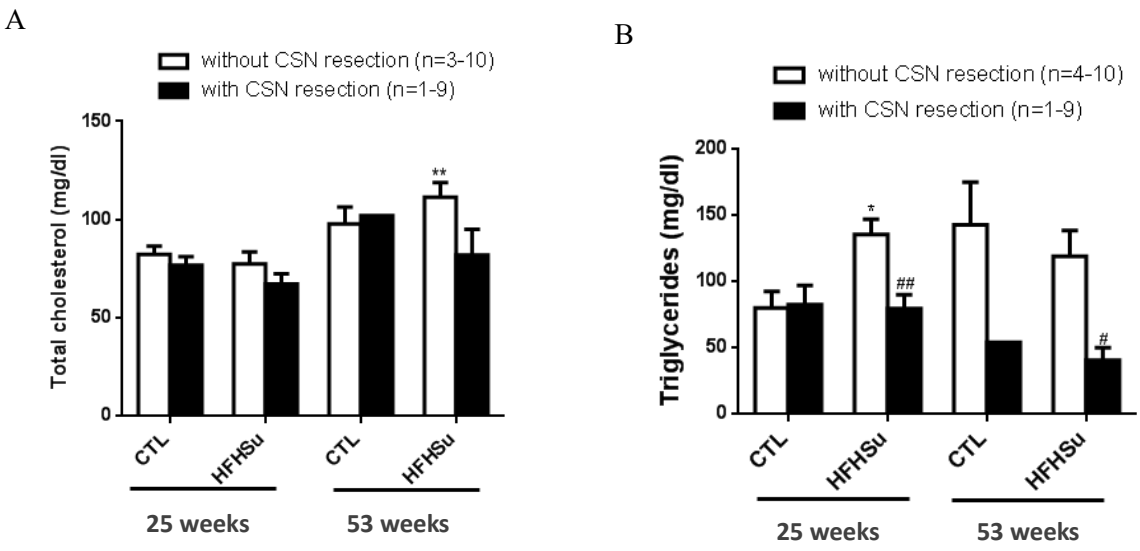

Figure 4. 4. Effect of long-term hypercaloric diet consumption and of carotid sinus nerve (CSN) resection on the plasmatic lipid profile of young and old animals: total cholesterol (A) and triglycerides (B) plasma levels. White bars represent the values of animals without CSN resection, black bars represent the values of animals with CSN resection. Bars represent mean±SEM of 1-10 values. One- and Two-Way ANOVA with Bonferroni multicomparison tests; *p<0.05 and**p<0.01 vs CTL 25 weeks; $^{\#}p$<0.05 and $^{\#\#}p$<0.01 comparing within the group.

4.8. Effect of long-term hypercaloric diet intake and carotid sinus nerve resection on total lipid and triglycerides content in the liver in young old animals

Figure 4.5. shows the effect of long-term hypercaloric diet intake and the effect of the chronic CSN resection on the liver´s lipid content in young and old animals. Ageing increased significantly by 101.35% lipid deposition in the liver. Also, hypercaloric diet consumption increase the percentage of total lipids in the liver by 47.97% and 83.56%,

in animals with 25 and 53 weeks of diet, respectively. CSN resection decreased lipid deposition in the liver in all groups of animals, an effect that was statistical significant in control (21.46% decrease) and aged (35.28% decrease) animals submitted to hypercaloric diets (Figure 4.5.).

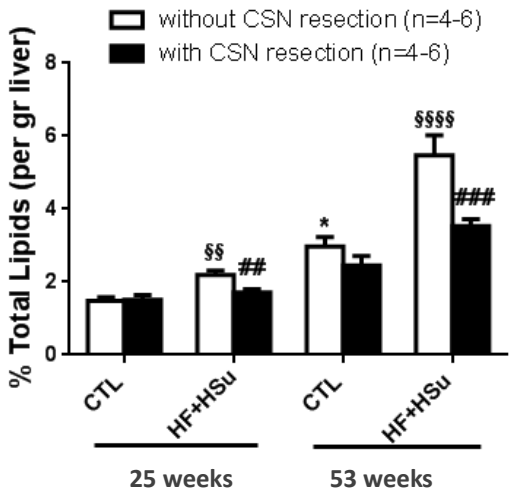

Figure 4. 5. Effect of long-term hypercaloric diet consumption and of carotid sinus nerve (CSN) resection on the level of total lipids content within the liver of young and old animals. White bars represent the values of animals without CSN resection, black bars represent the values of animals with CSN resection. Bars represent mean±SEM of 4-6 values. One- and Two-Way ANOVA with Bonferroni multicomparison tests; *p<0.05 vs CTL 25 weeks; ##p<0.01 and ###p<0.001 comparing within the group and §§p<0.01 and §§§§p<0.0001 comparing with controls with the same age.

Figure 4.6. shows the effect of long-term hypercaloric diet intake and the effect of the chronic CSN resection on the liver´s TG content in young and old animals. Ageing increased by 65.52% the TG accumulation in the liver, an effect that decreases in a non-significant manner with CSN resection. Hypercaloric diet consumption in young animals did not modify TG content in the liver, however age combined with the long-term hypercaloric consumption increased levels by 402.15%, an effect that was almost totally reversed with the CSN resection (57.13% decrease) (Figure 4.6.).

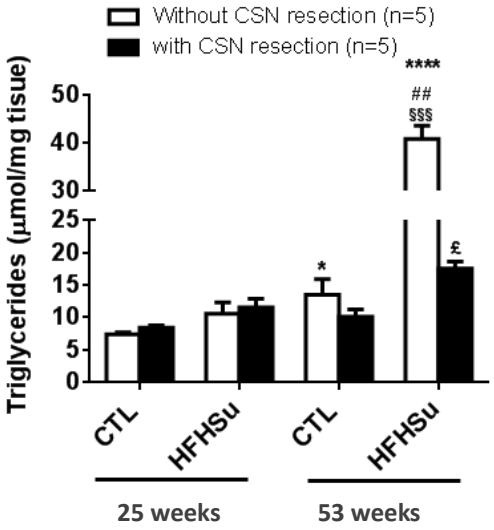

Figure 4. 6. Effect of long-term hypercaloric diet consumption and of carotid sinus nerve (CSN) resection on triglycerides levels within the liver of young and old animals. White bars represent the values of animals without CSN resection, black bars represent the values of animals with CSN resection. Bars represent mean±SEM of 5 values. One- and Two-Way ANOVA with Bonferroni multicomparison tests; *p<0.05 and ****p<0.0001 vs CTL 25 weeks; ##p<0.01 vs HFHSu 25 weeks; §§§p<0.001 comparing with controls with the same age and £p<0.05 comparing within the group.

4.8. Effect of long-term hypercaloric diet intake and carotid sinus nerve resection on hepatocellular changes in young old animals

Figure 4.7. depicts the effect of HFHSu diet, age and CSN resection on the macroscopic appearance of the liver. Liver images taken *post mortem* show an increase in lipid droplets with the intake of 25 and 53 weeks of HFHSu diet. Ageing also impacts lipid deposition within the liver, since older rats visually exhibit an increase in the lipid droplets. Ageing plus hypercaloric diet exacerbated lipid deposition in the liver. While, it was not possible to show the effects of the CSN resection in the young group, CSN denervation in HFHSu animals of 53 weeks clearly improved lipid deposition in the liver.

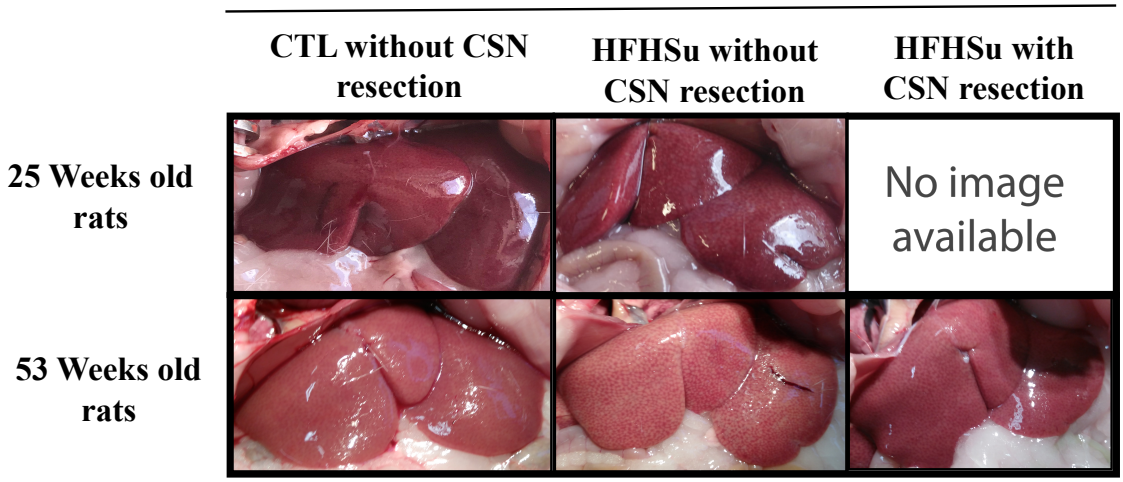

Figure 4. 7. Macroscopic images of the effect of long-term hypercaloric diet consumption and of carotid sinus nerve (CSN) resection in the liver of young and old animals.

To confirm the macroscopic data, a microscopic analysis of the livers was performed. H&E staining of the livers (Figure 4.8.) depict clear steatosis in animals submitted to the hypercaloric diet during 25 and 53 weeks. This steatosis was showed by marked liver micro and macrovesicular hepatocellular vacuolization. CSN resection improved these liver fatty changes both in young and old animals submitted to the HFHSu diet (Fig. 4.8). Age per se did not seem to alter significant lipid deposition as well as CSN denervation in control and aged animals fed with standard diet.

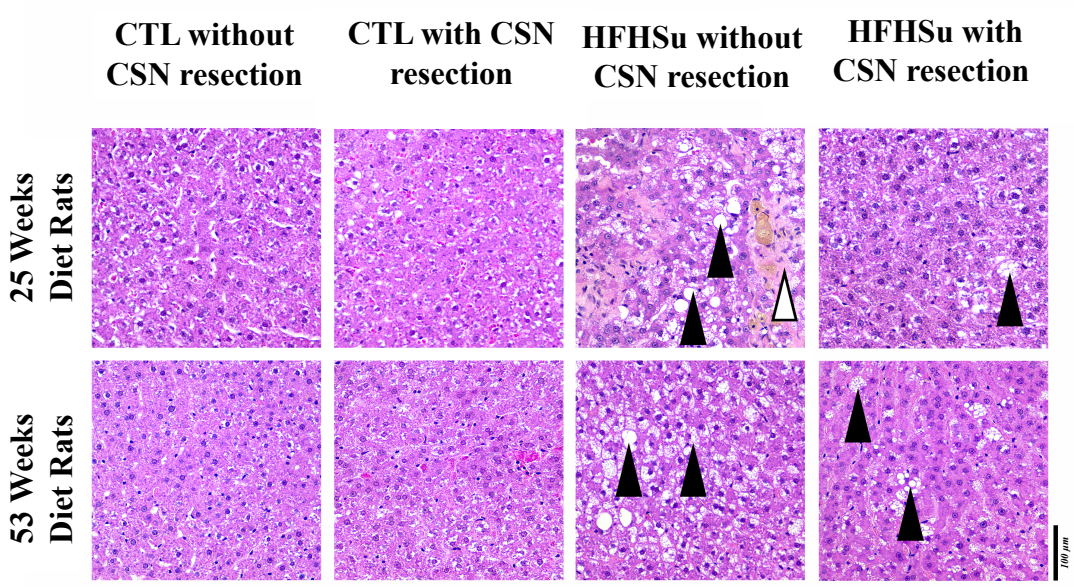

Figure 4. 8. Hematoxylin and Eosin (H&E) staining displaying the effect of long-term hypercaloric diet intake and the effect of chronic carotid sinus nerve (CSN) resection on the liver of young and old rats. Rats on HFHSu diet show marked hepatocellular vacuolization, micro and macrovesicular, which is reverted with CSN resection. Scale bar, 100 µm; original magnification 20x. Black arrowhead: fatty change (lipidosis); white arrowhead: fibrosis.

Figure 4.9. details the hepatocellular score analysis from the H&E staining displaying the effects of long-term hypercaloric diet intake and the effect of the chronic CSN resection on young and old animals. From the fatty change score (Figure 4.9. A) it was possible to observe an increase in tissue steatosis with diet consumption , an effect that was exacerbated in old animals. Also, CSN resection decreased fatty accumulation in HFHSu animals, this reaching statistical significance in old animals. Hepatocellular damage (Figure 4.9. B) increased in young HFHSu animals and in aged animals, these values reaching only significance in the old group. Note that CSN resection ameliorated or even reversed hepatocellular damage within these groups, respectively. Liver inflammation score (Figure 4.9. C) increased in young animals submitted to hypercaloric diet and in aged animals and CSN resection reversed these effects. In concordance with the inflammation score, the fibrosis score (Figure 4.9. D) increased in young animals submitted to hypercaloric diet and in aged animals, effects that were reduced with CSN resection.

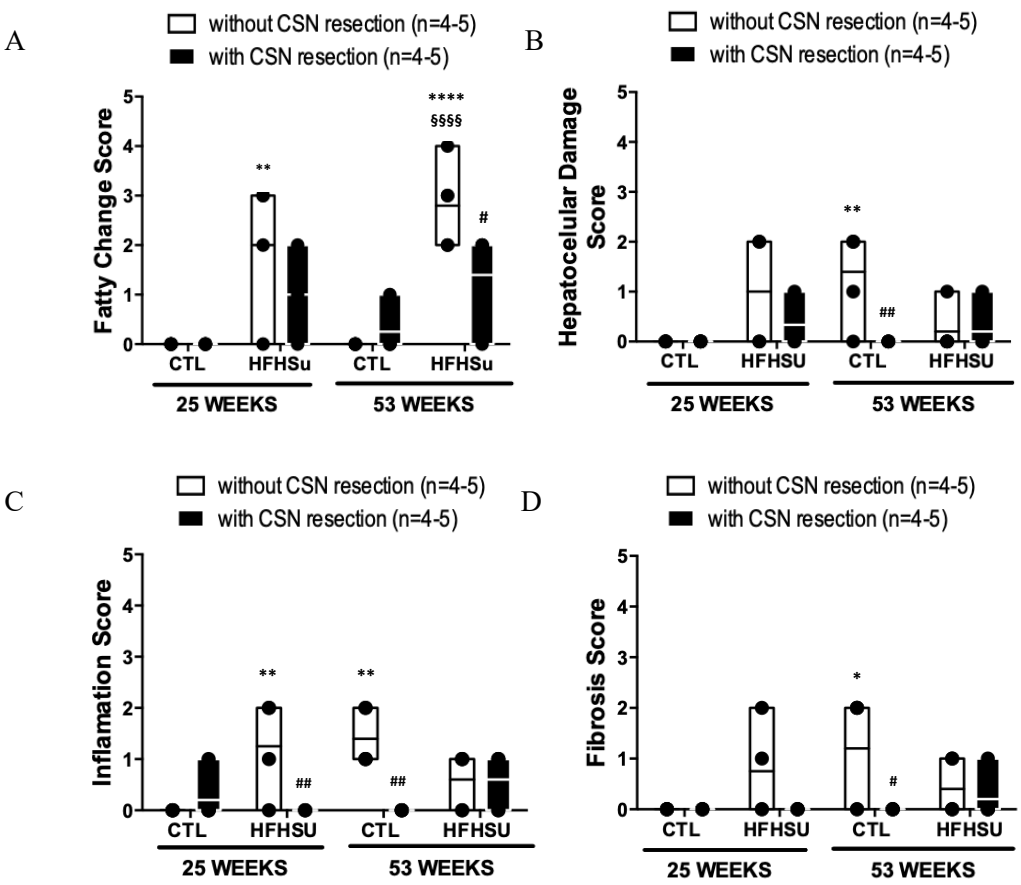

Figure 4. 9. Effect of long-term hypercaloric diet and of carotid sinus nerve (CSN) resection on the hepatocellular scores of young and old animals: fatty change (A), hepatocellular damage (B), inflammation (C)and fibrosis (D). White bars represent the values of animals without CSN resection, black bars represent the values of animals with CSN resection. Bars represent mean±SEM of 4-*5* values. One- and Two-Way ANOVA with Bonferroni multicomparison tests; **p*<0.05, ***p*<0.01 and *****p*<0.0001 vs CTL 25 weeks; #*p*<0.05 and ##*p*<0.01 comparing within the group and §§§§*p*<0.0001 comparing with controls with the same age.

5. Discussion and Conclusions

In this study, it was demonstrated that long-term hypercaloric consumption did not modify age-induced insulin resistance, while exacerbates glucose intolerance. It was also observed that the chronic bilateral CSN resection in old animals restored insulin sensitivity and glucose tolerance. Age and the HFHSu consumption increased plasma TG, an effect that was restored by the CSN resection. In the liver, the lipid content and the TG increased with the HFHSu diet and with age, being exacerbated by the long-term HFHSu consumption. Moreover, the CSN ameliorates theses alterations in the liver. We conclude that the CSN resection was able to restore insulin sensitivity and glucose tolerance and ameliorate liver steatosis induced by age and exacerbated by hypercaloric diet.

Age and the long-term HFHSu consumption did not modified the fasting glucose levels, an effect that was not altered by the CSN resection. These results are in accordance with previous works from Escrivá *et al* (2007). and Guarino *et al,* (2013) that did not observed changes in fasting glucose levels in Wistar rats with 3, 8, 12 and 24 months fed with a standard diet (Escrivá *et al.*, 2007; Guarino *et al.*, 2013). Also, Sacramento *et al*. did not described differences in the fasting glucose levels of CTL animals with 25 weeks of diet (Sacramento *et al.*, 2018). Shimokata *et al.* showed in an human cohort study that age leads to an increase in the fasting glucose levels and aging men tend to have higher levels than women (Shimokata *et al.*, 1991). Even though the fasting glucose remain unmodified with the long-term hypercaloric diet this effect was not observed in young animal models submitted to different hypercaloric diets. Melo *et al.* compared several groups of rats submitted to different types of hypercaloric diets for different periods of time: high-fat (HF) diets for 3 and 19 weeks, high-sucrose (HSu) diets for 4 and 16 weeks and with HFHSu diet for 25 weeks (Melo *et al.*, 2019) and demonstrated that all groups of animals increased their fasting glucose levels except the 16 weeks HSu animals (Melo *et al.*, 2019). The 3 weeks HF had an increase of 15%, the HF 19 weeks of 13%, HSu 4 weeks of 25% and HFHSu 25 weeks of 154% (Melo *et al.*, 2019). Even though fasting glucose levels increased in these animal models none of them reached values above 126 mg/dl, the reference value for T2D diagnosis. Chalkley et al. demonstrated in 2 months old Wistar rats fed for 9-10 months with HF diet that

there is an increase in the fasting glucose levels (Chalkley *et al.*, 2002). Kawasaki et al. showed in Wistar-Imamichi rats fed with Hsu diets for a total of 36 weeks and observed no difference in fasting glucose levels at 8, 12 and 24 weeks (Kawasaki *et al.*, 2005). However, after 28 and 36 weeks of HSu diet was observed an increase in fasting glycemia (Kawasaki *et al.*, 2005). Additionally, Sacramento *et al.* demonstrated that the increase in fasting glucose levels experienced by the 25 weeks HFHSu diet rats was reversed by the CSN resection (Sacramento *et al.*, 2018). Although, the increase in fasting glucose levels seen with the hypercaloric diets (HSu, HF and HFHSu) is extensively accepted, this effect was not seen in the animals submitted to 53 weeks of diet (Kamgang et al., 2005; Wilson and Hughes 1996; Ribeiro et al., 2005; Ebaidet al., 2006, Melo *et al.* 2019).

Age decreased insulin sensitivity, but the long-term HFHSu diet consumption did not intensify the age-induced insulin resistance. The decrease in the insulin sensitivity and the consequent peripheral insulin resistance with age, it is widely accepted as a consequence of age both in humans and animals (DeFronzo, 1981; Escrivá *et al.*, 2007; Fink *et al.*, 1983) . Additionally, Guarino *et al*. has also showed in rats with 3, 12 and 24 months increase insulin resistance with age (Guarino *et al.*, 2013). Knowing that age itself bring the drawback of insulin resistance both in animals and humans, it is of pivotal importance a way of oppose this tendency (DeFronzo, 1981; Escrivá *et al.*, 2007; Fink *et al.*, 1983).

Over the last years, it was demonstrated that the resection or the bioelectronic modulation of the CSN activity in young animal models of prediabetes and in an early-stage of T2D were able to prevent and reverse the insulin resistance and the glucose intolerance (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017, 2018). Knowing the social reality of an aging society, in the present work we tested the effect of CSN resection in old animals, as proof of principle that CSN/CB modulation might be a feasible therapeutic for these pathologies. We demonstrated for the first time that the CSN resection in aged CTL rats reverts the effects of aging on insulin sensitivity, an effect that was completely reversed at 4 weeks after the CSN resection. The denervation also reverted the insulin resistance in aged HFHSu (53 weeks of diet) animals, an effect that was achieved 2 weeks after the CSN resection, which counteracts the nefarious effects of the age and the HFHSu diet on their insulin sensitivity. CSN resection was also able

to restore insulin sensitivity in old animals. The increase in the SNS activity was proposed to be the link between the CB and the development of insulin resistance in young animals (Ribeiro *et al.*, 2013). Although in this work the SNS activity was not assessed, it has already been documented the deregulation of the SNS in older people (Esler *et al.*, 1995; Rowe and Troen, 1980).

One of the stepping points to the genesis of glucose intolerance is an already established insulin resistance. From the AUC of the OGTT it was observed that intolerant to glucose with 44 weeks of HFHSu diet, but the impact of age alone did not affect the glucose tolerance. Sacramento *et al.* showed that there is no alteration on the glucose tolerance in CTL rats with 25 weeks diet, however as expected animals fed with an HFHSu diet for a period of 25 weeks developed glucose intolerance. Therefore, the HFHSu diet consumption during 25 and 53 weeks have an impact on the glucose tolerance, which did not happen in the CTL animals, meaning that the caloric intake of the diets has a major role in the dysfunction of the glucose homeostasis. Other authors had also observed that the HF consumption during 14 and 17 weeks induced glucose intolerance in Wistar rats, in Sprague-Dawley rats and mice (Akiyama *et al.*, 1996; Marques *et al.*, 2016; Nascimento *et al.*, 2008; Winzell and Ahrén, 2004). Even though age did not alter the glucose tolerance in the CTL rats (13 months old), Wang *et al.* described in 22 months old Wistar rats a decrease in the tolerance to glucose and that the rats became glucose intolerant with age (Wang *et al.*, 1997). In this study, 13 months of age was enough to induce insulin resistance but not to induce glucose intolerance, meaning that probably if we increase the time of our experiments the animals might develop glucose intolerance (Wang *et al.*, 1997). Also, it is know that in humans an impaired glucose tolerance tends to develop with age (DeFronzo, 1981; Jackson, 1990). Yet, Reaven *et al*. made a great argument whether or not this age-related glucose intolerance seen in humans was a result of age *per se* or a secondary effect to other various age-related variables, as chronic diseases, medication and physically less active (Reaven and Reaven, 1985). So, it might be possible that some proportion of the decline in glucose tolerance may be independent of age in itself (Reaven and Reaven, 1985).

Furthermore, the old rats that became glucose intolerant with the HFHSu diet reverted this intolerance with the CSN resection. Therefore, the denervation of the CSN is not

only able to reverse the insulin resistance but also the glucose intolerance in old animals. This effect was also seen in the 25 week HFHSu diet animals, where the CSN resection and the bioelectronics modulation of the CSN activity was capable of reverting the glucose intolerance (Sacramento *et al.*, 2018). By comparing the previous results between 25 and 53 week HFHSu diet and the link between the SNS, the CB and the insulin resistance, a pattern for a possible therapy starts to arise, with the modulation of the CB or the CSN activity being a key part of it.

Herein, this work also showed that a long-term hypercaloric diet increases weight gain, as well as an increase in the rat' total fat depots: perienteric, perinephric and epididymal fat.

As expected caloric intake increases body weight, both in CTL animals fed a diet of 2.56 Kcal/g and in HFHSu 5.10 Kcal/g of high-fat diet plus 1.36 Kcal/g of high-sucrose. Even though the HFHSu animals were fed a much caloric diet there is only a slight increase in body weight. The animals may consume more calories but may burn more, as this animals might have been more active and spend more calories than the CTL. Also, the surgeries, sham and CSN resection, provoked a decrease in body weight. The decrease with the CSN resection is more pronounced than with the sham, so the decrease might not only be because of the surgery *per se* but also an effect of the CSN denervation. In animal studies has been sowed the increase of weight with an HF diet and a significant decrease with the CSN resection(Ribeiro *et al.*, 2013).

Age increased total fat in control animals when compared with animals submitted to 25 weeks of standard diet (Sacramento *et al.*, 2018), being this increase due to an augment in the perinephric and perienteric fat. CSN resection did not alter fat content in control old animals as it occurred in young control animals (25 weeks of diet) (Sacramento *et al.*, 2018). The increase in fat it is clearly marked with the 53 weeks of HFHSu diet. Sacramento *et al*. showed an increase in the fat depots HFHSu animals submitted to 25 weeks of diet. Even though, Sacramento *et al*. observed a decrease in the perinephric fat with the CSN resection, the old animals did not show a decrease in any of the fat depots with the CSN resection. One possible explanation is that the amount accumulated during the 53 weeks on hypercaloric diet requires more time to have an effect than a period of nine weeks after the denervation. A similar situation might occur on the 25 weeks of diet animals where eleven weeks of follow-up it is not

enough to see marked changes in the fat depots. Moreover, the increase in fat content described with the hypercaloric diet brings some health complications, like obesity and dyslipidemia and aggravates the insulin resistance (Kim *et al.*, 2000). There is a correlation between the decrease in insulin sensitivity and the increase in body fat deposition in animals as well as in humans (Carey *et al.*, 1996; Kim *et al.*, 2000). In rats and mice fed with a HF diet it was observed an increase in visceral fat accumulation, whole body and muscle insulin resistance and hyperinsulinemia within 4 week (Han *et al.*, 1997; Kim *et al.*, 2000; Kraegen *et al.*, 1986; Zierath *et al.*, 1997) . Additionally, if the HF diet continues for a long period of time (8 months), rats and mice develop severe visceral obesity and diabetes or impaired glucose tolerance (Han *et al.*, 1997; Kim *et al.*, 2000). Therefore, the increase in fat depots may influence the decrease in insulin sensitivity seen in the old and young animals.

Total cholesterol levels in plasma did not change significantly with age. It was previously described that cholesterol levels tend to increase with age both in humans and animals (Uchida *et al.*, 1978). Uchida *et al*. demonstrated that serum and liver lipid levels increase with age in both Sprague-Dawley and Wistar rats (Uchida *et al.*, 1978). Also, plasma levels of total and LDL-cholesterol are well known to increase with aging (Uranga and Keller, 2010). This might be a casual factor of the decrease in the plasma clearance of LDL in humans and rodents, the decreased breakdown of cholesterol to bile acids in aging rodents, as well as the increased intestinal cholesterol absorption in aging mice and rats (Ericsson *et al.*, 1991; Uranga and Keller, 2010; Wang, 2002). Additionally, total cholesterol increased in old HFHSu animals, an effect that was not observed in young HFHSu animals, which leads to the conclusion that the increase observed in old animals is a result of the long-term hypercaloric intake. The hypercaloric diets consumption specially during a prolonged period tends to increase plasma total cholesterol levels in rats, since the caloric intake effects poorly the lipid profile (Uranga and Keller, 2010).

The effects of HF diets on cholesterol metabolism have been studied in both humans and animals, and it have been associated with hyperlipidemia (Uranga and Keller, 2010). Studies in humans have shown that Western diets consumption induced an increase in both total and LDL cholesterol levels in plasma and a significant reduction in HDL cholesterol (Uranga and Keller, 2010) . When people consume a balanced diet

and low in fat exhibit low total cholesterol and low LDL cholesterol levels (McMurry *et al.*, 1991; Uranga and Keller, 2010). However, when these people are placed on more typical Western diets, their total and LDL cholesterol levels increase (McMurry *et al.*, 1991; Uranga and Keller, 2010).

In this study, it was observed an increase in TG levels in control old animals. The increase in TG with age is not consensual in the literature. Mesomya *et al.* showed in Sprague – Dawley rats of 4, 8 and 12 months an irregular variation of TG levels with age, since the levels of TG increased from 4 to 8 months but decreased from 8 to 12 months (Mesomya *et al.*, 2001). However, high TG levels have been demonstrated in aged rats (Jayachandran *et al.*, 1996). In relation to human data, a study by Puga *et al.* was unable to find changes in TG levels between young and old people (Puga *et al.*, 2011) but Cassader *et al*. showed that older people have higher TG levels (Cassader *et al.*, 1996).

In the present thesis, TG levels in old HFHSu animals were similar than TG levels in young HFHSu animals, suggesting that long-term hypercaloric diet did not exacerbate the increase in TG levels that it is established with 25 weeks of hypercaloric diet. The absence of statistical significance in the old animals might be due to the reduced number of samples (n=4). Although our results are in agreement with the literature: Yan *et al*. described in Wistar rats fed for 20 weeks with HF diet an increase in the TG plasma levels (Yan *et al.*, 2006) and Melo *et al*. also demonstrated the increase the triglycerides levels in HF 3 weeks, HF 19 weeks, Hsu 4 weeks, HSu 16 weeks and HFHSu 25 weeks of diet animals (Melo *et al.*, 2019). CSN resection in 25 and 53 week HFHSu rats significantly impact TG levels, as the values were significantly reduced, elucidating once more the beneficial effects of CB activity ablation.

Metabolic diseases are connected to each other, through the disturbance of lipids in the body and insulin resistance (Blaton, Korita and Bulo, 2008; Katsiki, Mikhailidis and Mantzoros, 2016; Klop, Elte and Cabezas, 2013; Mooradian, 2009). Since, insulin resistance influences the increase in FFA and the deregulation of the lipid metabolism in the liver and adipose tissue (Katsiki, Mikhailidis and Mantzoros, 2016), and knowing from the already discussed results of the effect of the CSN denervation on the insulin resistance, it is plausible that the CSN resection impacts the rats lipid profile through the improvement on insulin sensitivity.

The liver is one of the central organs in metabolic diseases and therefore the present study aimed to elucidate the effects of the age and long-term HFHSu diet and the CSN resection on the liver dysfunction of young and old animals. A macroscopic evaluation of the livers at a terminal experiment, showed a slight increase in lipidic droplets in aged animals and in animals submitted to hypercalorioc diet and an exacerbated effect when diet is combined with age . Even though there is no visual record of the effect of the CSN resection on the liver of young HFHSu animals, the liver from HFHSu old animals submitted to the CSN resection showed a high reduction in these lipidic droplets.

The total lipid and TG content in the liver as evaluated to confirm the macroscopic results. From this analysis, it was possible to observe an increase in the total lipid content with the age and with the hypercaloric diet in both young and old groups. The TG content increased with age and the long-term HFHSu diet. Both in rats and humans age leads to an increase in the lipids accumulation in the liver (Castro, de *et al.*, 2013; Kuk *et al.*, 2009). Age can also create dysfunctions in the lipid and glucose metabolism (Guarino *et al.*, 2013; Uchida *et al.*, 1978). Moreover, the increase in the total lipid content and TG was higher in the animals submitted to the long-term HFHSu diet. As, Westerbacka et al. demonstrated in humans, the type of diet has a key role in the lipid accumulation in the liver, since liver fat decrease during consumption of low-fat diet and increase with HF diet (Westerbacka *et al.*, 2005). Knowing that hypercaloric diet leads to an increase in the lipid profile, which is deeply connected to insulin resistance and metabolic diseases, this can bring considerable consequences for the liver(Taskinen, 2005; Uranga and Keller, 2010; Yan *et al.*, 2006). Moreover, since a dysfunction in glucose homeostasis and dyslipidemia can create a flow of FFA into the liver, these dysfunctions induced by the hypercaloric diets or aging might lead to the eventual hepato-steatosis (Vanni *et al.*, 2010).

An impaired glucose uptake by the muscle and an inadequate insulin suppression of lipolysis in the adipose tissue, together with the liver´s overproduction of glucose, despite fasting hyperinsulinemia, contribute greatly to the increase of fat deposition in the liver and may be the reason behind the increase of total lipid content in the liver and of the increase in lipidic droplets observed both, macroscopic and microscopically in

liver images (Vanni *et al.*, 2010; Yki-Järvinen, 1993). Being the lipolysis the main source of hepatic TG in NAFLD, an peripheral insulin resistance would result in the increase of the TG deposition in the liver, as seen in the old animals (Vanni *et al.*, 2010; Yki-Järvinen, 1993). Studies have suggested that increased delivery of FFA into the liver is associated with increase of liver fat content, as the one observed in the old animals(Taskinen, 2005). The increase in lipid accumulation in the liver leads to the hepato-steatosis seen metabolic diseases like NAFLD, which can then evolve into NASH (Bugianesi *et al.*, 2005; Seppälä-Lindroos *et al.*, 2002).

Herein we found that CSN resection not only improved the overall body glucose metabolism but also improve dysfunction (adipose tissue, muscle and liver) and impairs normal metabolic processes (glucose uptake, lipolysis and hepatic *de novo* lipogenesis inhibition) (Bajaj and DeFronzo, 2003; Boden and Chen, 1995; Kashyap *et al.*, 2003). So, by restoring insulin sensitivity the overall improvement can lead to the hepatocellular restoration. The reduction of the lipid accumulation with the CSN resection might be an indirect effect, due to the restoration of insulin sensitivity or might be a more direct effect on the liver. In order to elucidate this further studies are required.

Confirming our macroscopic and biochemical alterations in the liver, the microscopic H&E staining analysis of the liver showed slight differences with age. As expected, an increase in the lipidosis was observed in both young and old animals fed with hypercaloric diets and a decrease, as seen earlier, with the CSN resection. Aging is associated with a physiological increase in lipid accumulation in the liver and with an increased prevalence of NAFLD in aged humans(Sheedfar *et al.*, 2013). Due to an increase in hypercaloric food availability and consumption the prevalence of NAFLD has increase (Alwahsh and Gebhardt, 2017). The hepatocellular scores were performed in order to accessed if the effects of the hypercaloric diet and the age were able to induce other NAFLD characteristics. According to the "two-hits" model for the progression of liver damage, the "first hit" is marked by a reversible deposition of lipids in the liver, and the "second hit" by irreversible hepatocyte injury, like lipid peroxidation, induction of inflammatory cells and fibrogenesis with extracellular matrix deposition(Vanni *et al.*, 2010). Clearly, the animal model studied in the present work was capable of mimic the "first hit" of NAFLD with the deposition of lipids in the liver.

When the livers were analyzed for the fatty change score, to evaluate the "first-hit" of NAFLD, no differences were found with age, but it was observed an increase in lipids accumulation with HFHSu diets in both young and old groups and a decrease with the CSN denervation in the HFHSu old animals, confirming the macroscopic images and the liver lipid content. The hepatocellular damage score evaluates the potential tissue hyperplasia or hypertrophy, which are major contributors of the progression from NAFLD to NASH, and therefore the "second hit" of NAFLD. In the present work, we observed an increase in the hepatocellular damage with age and HFHSu diet (25 weeks) but not with the long-term hypercaloric diet. These results were quite surprisingly, but can anticipate a possible compensation to avoid major liver dysfunction. CSN resection was able to reduce the cellular damage in the liver of old control animals. Tissue inflammation is a step forward on the progression of the NAFLD that drives the transition from benign steatosis toward more advanced NASH(Sheedfar *et al.*, 2013). Livers were analyzed for the liver's inflammation score and it was possible to observe an increase with age and with the 25 weeks hypercaloric diet, effects that were restored by the CSN denervation. Fibrosis is seen as a defining characteristic of the NASH and represents an irreversible evolution of the pathology (Vanni *et al.*, 2010). The fibrosis score showed an increase with age and with the hypercaloric diet, an effect that was reverted with the CSN resection.

All these results combined lead us to believe that this model is capable of simulating the disease steps in humans. The early stage of the disease is marked by an increasing lipidosis in the liver, as showed in the livers of the 25 weeks HFHSu diet rats. Importantly, this model not only shows the early stage of the disease but also a progression, observed by the hepatocellular damage and tissue inflammation, into a more severe stage of the disease. Herein, some old animals and some animals submitted to 25 weeks of HFHSu diet start to show also evidences of the late stage of NAFLD, NASH, which is deeply marked with tissue fibrosis and with considered irreversible damage (Vanni *et al.*, 2010).

Moreover, these results showed that the ablation of the activity of the CB/CSN has a great potential to treat NAFLD and even NASH, two disorders that lack therapeutic strategies, and which the usual outcome is the liver transplant (Vanni *et al.*, 2010). So, further investigation is required to elucidate the link between the CB, SNS, insulin

resistance and metabolic disturbances, like NAFLD and to understand if the effects seen in this work are from a direct modulation on the CB into the organ and/or a result of an indirect approach.

In conclusion, in an aged world with increasingly incidence of metabolic diseases and where disorders like MS, NAFLD, T2D and prediabetes are seen as a growing epidemic it is of outmost importance to find a way to stop these disorders. Only with an efficient therapy we can oppose this public health issue. With the study of the mechanisms behind these comorbidities we might uncover an approach to stop the pathological vicious cycle. The present study, demonstrates that the abolishment of the CB/CSN activity in old rats can restore insulin sensitivity, glucose tolerance and have significant impact on the liver of these animals. Our results suggest that the modulation of CB activity might be important in age-induced metabolic dysfunction and metabolic dysfunction exacerbated by hypercaloric diet consumption, but also in liver-associated metabolic dysfunctions in young and old animals. So, this work might have shed some light on a possible therapeutic strategy with the modulation of the CB or the CSN activity to not only treat but also to prevent these age-associated disorders.

5. References

ADIELS, M. *et al.* - Overproduction of large VLDL particles is driven by increased liver fat content in man. **Diabetologia**. . ISSN 0012186X. 49:4 (2006) 755–765. doi: 10.1007/s00125-005-0125-z.

ADIELS, M. *et al.* - Acute suppression of VLDL1 secretion rate by insulin is associated with hepatic fat content and insulin resistance. **Diabetologia**. . ISSN 0012186X. 50:11 (2007) 2356–2365. doi: 10.1007/s00125-007-0790-1.

AKIYAMA, Toshiharu *et al.* - High-fat hypercaloric diet induces obesity, glucose intolerance and hyperlipidemia in normal adult male Wistar rat. **Diabetes Research and Clinical Practice**. . ISSN 01688227. 31:1996) 27–35. doi: 10.1016/0168-8227(96)01205-3.

ALBERTI, K. G.; ZIMMET, P.; SHAW, JE - The Metabolic Syndrome - A New Worldwide Definition. **Lancet**. . ISSN 1474-547X. 366:2005) 1059–1062. doi: 10.1016/S0140-6736(05)67402-8.

ALWAHSH, Salamah Mohammad; GEBHARDT, Rolf - Dietary fructose as a risk factor for non-alcoholic fatty liver disease (NAFLD). **Archives of Toxicology**. . ISSN 14320738. 91:4 (2017) 1545–1563. doi: 10.1007/s00204-016-1892-7.

ARNER, Peter - Human fat cell lipolysis: Biochemistry, regulation and clinical role. **Best Practice and Research: Clinical Endocrinology and Metabolism**. . ISSN 1521690X. 19:4 (2005) 471–482. doi: 10.1016/j.beem.2005.07.004.

ATANASOVA, Dimitrinka Y.; ILIEV, Michail E.; LAZAROV, Nikolai E. - Morphology of the rat carotid body. **Biomedical Reviews**. . ISSN 1310392X. 22:December (2011) 41–55. doi: 10.14748/bmr.v22.34.

BAJAJ, Mandeep; DEFRONZO, Ralph A. - Metabolic and molecular basis of insulin resistance. **Journal of Nuclear Cardiology**. . ISSN 10713581. 10:3 (2003) 311–323. doi: 10.1016/S1071-3581(03)00520-8.

BARBIERI, Michelangela *et al.* - Age-related insulin resistance: Is it an obligatory finding? The lesson from healthy centenarians. **Diabetes/Metabolism Research and Reviews**. . ISSN 15207552. 17:1 (2001) 19–26. doi: 10.1002/dmrr.178.

BARZILAI, Nir *et al.* - The critical role of metabolic pathways in aging. **Diabetes**. . ISSN 00121797. 61:6 (2012) 1315–1322. doi: 10.2337/db11-1300.

BERGMAN, R. N. - Non-esterified fatty acids and the liver: why is insulin secreted into the portal vein? **Diabetologia**. . ISSN 00304026. 43:2000) 946–952. doi: 10.1016/j.ijleo.2015.08.155.

BIN-JALIAH, I.; MASKELL, P. D.; KUMAR, P. - Indirect sensing of insulin-induced hypoglycaemia by the carotid body in the rat. **Journal of Physiology**. . ISSN 00223751. 556:1 (2004) 255–266. doi: 10.1113/jphysiol.2003.058321.

BLATON, Victor H.; KORITA, Irena; BULO, Anyla - How is metabolic syndrome related to dyslipidemia ? **Biochemia Medica**. 18:1 (2008) 14–24.

BODEN, Guenther *et al.* - Effects of free fatty acids on gluconeogenesis and autoregulation of glucose production in type 2 diabetes. **Diabetes**. . ISSN 00121797. 50:4 (2001) 810–816. doi: 10.2337/diabetes.50.4.810.

BODEN, Guenther; CHEN, Xinhua - Effects of fat on glucose uptake and utilization in patients with non-insulin-dependent diabetes. **Journal of Clinical Investigation**. . ISSN 00219738. 96:3 (1995) 1261–1268. doi: 10.1172/JCI118160.

BODEN, Guenther; SHULMAN, G. I. - Free fatty acids in obesity and type 2 diabetes: Defining their role in the development of insulin resistance and β-cell dysfunction. **European Journal of Clinical Investigation**. . ISSN 00142972. 32:SUPPL. 3 (2002) 14–23. doi: 10.1046/j.1365-2362.32.s3.3.x.

BONOMINI, Francesca; RODELLA, Luigi Fabrizio; REZZANI, Rita - Metabolic Syndrome, Aging and Involvement of Oxidative Stress. **Aging and Disease**. . ISSN 2152-5250. 6:2 (2015) 109–120. doi: 10.14336/ad.2014.0305.

BRUNO, Graziella *et al.* - Incidence of type 1 and type 2 diabetes in adults aged 30-49 years. **Diabetes Care**. . ISSN 01495992. 28:11 (2005) 2613–2619. doi: 10.2337/diacare.28.11.2613.

BUGIANESI, E. *et al.* - Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: Sites and mechanisms. **Diabetologia**. . ISSN 0012186X. 48:4 (2005) 634–642. doi: 10.1007/s00125-005-1682-x.

CAREY, David G. *et al.* - Abdominal fat and insulin resistance in normal and overweight women: Direct measurements reveal a strong relationship in subjects at both low and high risk of NIDDM. **Diabetes**. 45:5 (1996) 633–638.

CARO, J. F. *et al.* - Insulin receptor kinase in human skeletal muscle from obese subjects with and without noninsulin dependent diabetes. **Journal of Clinical Investigation**. . ISSN 00219738. 79:5 (1987) 1330–1337. doi: 10.1172/JCI112958.

CASSADER, M. *et al.* - Postprandial triglyceride-rich lipoprotein changes in elderly and young subjects. **Aging Clinical and Experimental Research**. . ISSN 03949532. 8:6 (1996) 421–428.

CASTRO, Uberdan Guilherme Mendes DE *et al.* - Age-dependent effect of high-fructose and high-fat diets on lipid metabolism and lipid accumulation in liver and kidney of rats. **Lipids in Health and Disease**. . ISSN 1476-511X. 12:1 (2013) 136. doi: 10.1186/1476-511X-12-136.

CATALANO, Karyn J.; BERGMAN, Richard N.; ADER, Marilyn - Increased Susceptibility to Insulin Resistance Associated with Abdominal Obesity in Aging Rats. **Obesity Research**. . ISSN 10717323. 13:1 (2005) 11–20. doi: 10.1038/oby.2005.4.

CHALKLEY, Simon M. *et al.* - Long-term high-fat feeding leads to severe insulin resistance but not diabetes in Wistar rats. **American Journal of Physiology - Endocrinology and Metabolism**. . ISSN 01931849. 282:2002) 1231–1238.

CHATRATH, Hemant; VUPPALANCHI, Raj; CHALASANI, Naga - Dyslipidemia in patients with nonalcoholic fatty liver disease. **Seminars in Liver Disease**. . ISSN 02728087. 32:1 (2012) 22–29. doi: 10.1055/s-0032-1306423.

CHERRINGTON, Alan D. - Control of Glucose Uptake and Release by the Liver In Vivo. **Diabetes**. . ISSN 0012-1797. 48:1999) 1198–1214. doi: 10.2337/diabetes.48.5.1198.

COLVILLE, C. A. *et al.* - Kinetic analysis of the liver-type (GLUT2) and brain-type (GLUT3) glucose transporters in Xenopus oocytes: Substrate specificities and effects of transport inhibitors. **Biochemical Journal**. . ISSN 02646021. 290:3 (1993) 701–706. doi: 10.1042/bj2900701.

CONDE, Silvia V. *et al.* - Function of the rat carotid body chemoreceptors in ageing. **Journal of Neurochemistry**. . ISSN 00223042. 99:3 (2006) 711–723. doi: 10.1111/j.1471-4159.2006.04094.x.

CONDE, Sílvia V. *et al.* - Carotid body, insulin and metabolic diseases: Unravelling the links. **Frontiers in Physiology**. . ISSN 1664042X. 5:OCT (2014) 1–14. doi: 10.3389/fphys.2014.00418.

CONDE, Silvia V.; SACRAMENTO, Joana F.; GUARINO, Maria P. - Carotid body: a metabolic sensor implicated in insulin resistance. **Physiological Genomics**. . ISSN 1094-8341. 50:3 (2018) 208–214. doi: 10.1152/physiolgenomics.00121.2017.

CORNIER, Marc-Andre *et al.* - The metabolic syndrome. **Endocrine Reviews**. . ISSN 0032745X. 29:7 (2008) 777–822. doi: 10.1210/er.2008-0024.

CUSI, Kenneth *et al.* - Insulin resistance differentially affects the PI 3-kinase– and MAP kinase–mediated signaling in human muscle. **The Journal of Clinical Investigation**. 105:3 (2000) 311–320.

DAY, C. P. - Natural History of NAFLD: Remarkably Benign in the Absence of Cirrhosis. **Gastroenterology**. . ISSN 00165085. 129:1 (2005) 375–378. doi: 10.1053/j.gastro.2005.05.042.

DAY, C. P.; JAMES, O. F. W. - Steatohepatitis: A tale of two «Hits»? **Gastroenterology**. . ISSN 00165085. 114:4 I (1998) 842–845. doi: 10.1016/S0016-5085(98)70599-2.

DEFRONZO, Ralph A. - Glucose Intolerance and Aging. **Diabetes Care**. 4:4 (1981) 493–501.

DEFRONZO, Ralph A. *et al.* - Effects of insulin on peripheral and splanchnic glucose metabolism in Noninsulin-depencent (Type II) Diabetes Mellitus. **Hepatic and Peripheral Glucose Metabolism in Noninsulin-dependent Diabetes**. 76:1985) 149–155.

DEFRONZO, Ralph A. - Pathogenesis of type 2 diabetes mellitus. **Medical Clinics of North America**. . ISSN 00257125. 88:4 (2004) 787–835. doi: 10.1016/j.mcna.2004.04.013.

DEFRONZO, Ralph A. *et al.* - Type 2 Diabetes Mellitus. **Nature Reviews: Disease Primers**. 1:2015) 255–272. doi: 10.1016/B978-0-12-803376-0.00012-5.

DEFRONZO, Ralph A.; BONADONNA, Riccardo C.; FERRANNINI, Eleuterio - Pathogenesis of NIDDM: A Balanced Overview. **Diabetes care**. . ISSN 01688227. 15:3 (1992) 318–368. doi: 10.1016/0168-8227(88)90005-8.

DEFRONZO, Ralph A.; TRIPATHY, Devjit - Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. **Diabetes care**. . ISSN 19355548. 32 Suppl 2:2009) s157–s163. doi: 10.2337/dc09-S302.

DEVERS, Marion C.; CAMPBELL, Stewart; SIMMONS, David - Influence of age on the prevalence and components of the metabolic syndrome and the association with cardiovascular disease. **BMJ Open Diabetes Research and Care**. . ISSN 20524897. 4:1 (2016) 1–7. doi: 10.1136/bmjdrc-2016-000195.

DYMECKA, A.; WALSKY, M.; POKORSKI, M. - ULTRASTRUCTURAL DEGRADA TION OF THE CAROTID BODY IN THE AGED RA T : IS THERE A ROLE FOR A THEROSCLEROSIS IN THE MAIN CAROTID ARTERIES ? **JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY**. 57, supp 4:2006) 85–90.

EGAN, Brent M. - Insulin Resistance and the Sympathetic Nervous System. **Vnitrni Lekarstvi**. . ISSN 0042-773X. 5:2003) 247–254.

EISENBERG, Michael L. *et al.* - Insulin receptor (IR) and glucose transporter 2 (GLUT2) proteins form a complex on the rat hepatocyte membrane. **Cellular Physiology and Biochemistry**. . ISSN 10158987. 15:2005) 51–58. doi: 10.1159/000083638.

ENNS, Gregory - Metabolic disease. Em Encyclopedia Britannica. [S.l.] : Encyclopedia Britannica, inc, 2019

ERICSSON, Sverker *et al.* - Influence of age on the metabolism of plasma low density lipoproteins in healthy males. **Journal of Clinical Investigation**. . ISSN 00219738. 87:2 (1991) 591–596. doi: 10.1172/JCI115034.

ESCRIVÁ, Fernando *et al.* - Effect of age and moderate food restriction on insulin sensitivity in Wistar rats: Role of adiposity. **Journal of Endocrinology**. . ISSN 00220795. 194:1 (2007) 131–141. doi: 10.1677/joe.1.07043.

ESLER, M. D. *et al.* - Aging effects on human sympathetic neuronal function. **American Journal of Physiology - Regulatory Integrative and Comparative Physiology**. . ISSN 03636119. 268:1995) R278–R285.

ESLER, Murray *et al.* - Mechanisms of sympathetic activation in obesity-related hypertension. **Hypertension**. . ISSN 0194911X. 48:5 (2006) 787–796. doi: 10.1161/01.HYP.0000242642.42177.49.

FABBRINI, Elisa *et al.* - Alterations in Adipose Tissue and Hepatic Lipid Kinetics in Obese Men and Women With Nonalcoholic Fatty Liver Disease. **Gastroenterology**. . ISSN 00165085. 134:2 (2008) 424–431. doi: 10.1053/j.gastro.2007.11.038.

FADINI, Gian Paolo *et al.* - At the crossroads of longevity and metabolism: The metabolic syndrome and lifespan determinant pathways. **Aging Cell**. . ISSN 14749718. 10:1 (2011) 10–17. doi: 10.1111/j.1474-9726.2010.00642.x.

FINK, Raymond I. *et al.* - Mechanisms Resistance Aging. **The Journal of Clinical Investigation**. 71:June (1983) 1523–1535.

FINKEL, Toren - The metabolic regulation of aging. **Nature Medicine**. 21:2015) 1416–1423.

FINKEL, Toren; HOLBROOK, Nikki J. - Oxidants, oxidative stress and the biology of ageing. **Nature**. 408:November (2000) 239–247.

FIRTH, R. G. *et al.* - Postprandial hyperglycemia in patients with noninsulin-dependent diabetes mellitus. **Journal of Clinical Investigation**. . ISSN 00219738. 77:5 (1986) 1525–1532. doi: 10.1172/JCI112467.

FOLCH, Jordi; LEES, M.; SLOANE STANLEY, G. .. - A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES. **j. Biol. Chem.** . ISSN 0021-9258. 226:1957) 497–509.

FOLSOM, Aaron R. *et al.* - Body Fat Distribution and 5-Year Risk of Death in Older Women. **JAMA**. 269:4 (1993) 483–487.

FOROUZANFAR, M. H.; ALEXANDER, L.; ANDERSON, H. R. - Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. **Lancet**. 386:10010 (2015) 2287–2323. doi: 10.1016/S0140-6736(15)00128-2.

FRAYN, Keith N. - Adipose tissue and the insulin resistance syndrome. **Proceedings of the Nutrition Society**. . ISSN 0029-6651. 60:3 (2001) 375–380. doi: 10.1079/pns200195.

GALLEGO-MARTIN, Teresa *et al.* - Effects of low glucose on carotid body chemoreceptor cell activity studied in cultures of intact organs and in dissociated cells. **American Journal of Physiology-Cell Physiology**. . ISSN 0363-6143. 302:8 (2012) C1128–C1140. doi: 10.1152/ajpcell.00196.2011.

GARDETE-CORREIA, L. *et al.* - First diabetes prevalence study in Portugal: PREVADIAB study. **Diabetic Medicine**. . ISSN 07423071. 27:8 (2010) 879–881. doi: 10.1111/j.1464-5491.2010.03017.x.

GERICH, John E. *et al.* - Renal gluconeogenesis. **Diabetes care**. . ISSN 00652571. 24:2 (2001) 382–391. doi: 10.1016/0065-2571(63)90034-7.

GIROD, John P.; BROTMAN, Daniel J. - The metabolic syndrome as a vicious cycle: Does obesity beget obesity? **Medical Hypotheses**. . ISSN 03069877. 60:4 (2003) 584–589. doi: 10.1016/S0306-9877(03)00053-7.

GIULIO, C. Di *et al.* - Physiological Carotid Body Denervation During Aging. **The Peripheral Nervous System**. 1974) 257–263. doi: 10.1007/978-1-4615-8699-9_18.

GIULIO, C. DI *et al.* - Selected contribution: Carotid body as a model for aging studies: is there a link between oxygen and aging? **Journal of applied physiology (Bethesda, Md. : 1985)**. . ISSN 87507587. 95:4 (2003) 1755–1758. doi: 10.1152/japplphysiol.00406.2003.

GIULIO, Camillo Di *et al.* - Human Carotid Body HIF and NGB Expression During Human Development and Aging. **Journal of the Neurological Sciences**. . ISSN 0022510X. 101:2 (2012) 265–271. doi: 10.1016/0022-510x(91)90053-a.

GONZALEZ, C. *et al.* - **Carotid body chemoreceptors: from natural stimuli to sensory discharges.** ISBN 0031933319.

GONZÁLEZ, Constancio *et al.* - Cellular mechanisms of oxygen chemoreception in the carotid body. **Respiration Physiology**. . ISSN 00345687. 102:1995) 137–147. doi: 10.1016/0034-5687(95)00069-0.

GRASSI, G. *et al.* - Neuroadrenergic and reflex abnormalities in patients with metabolic syndrome. **Diabetologia**. . ISSN 0012186X. 48:7 (2005) 1359–1365. doi: 10.1007/s00125-005-1798-z.

GRASSI, Guido *et al.* - Excessive sympathetic activation in heart failure with obesity and metabolic syndrome: Characteristics and mechanisms. **Hypertension**. . ISSN 0194911X. 49:3 (2007) 535–541. doi: 10.1161/01.HYP.0000255983.32896.b9.

GRUNDY, Scott M. - What is the contribution of obesity to the metabolic syndrome? **Endocrinology and Metabolism Clinics of North America**. . ISSN 08898529. 33:2 (2004) 267–282. doi: 10.1016/j.ecl.2004.03.001.

GRUNDY, Scott M. *et al.* - Diagnosis and management of the metabolic syndrome in obesity. **Obesity Reviews**. . ISSN 14677881. 6:4 (2005) 283–296. doi: 10.1111/j.1467-789X.2005.00221.x.

GRUNDY, Scott M. - Metabolic syndrome update. **Trends in Cardiovascular Medicine**. . ISSN 18732615. 26:4 (2016) 364–373. doi: 10.1016/j.tcm.2015.10.004.

GUARINO, Maria P. *et al.* - Chronic caffeine intake reverses age-induced insulin resistance in the rat: Effect on skeletal muscle Glut4 transporters and AMPK activity. **Age**. . ISSN 01619152. 35:5 (2013) 1755–1765. doi: 10.1007/s11357-012-9475-x.

GUARNER-LANS, Verónica *et al.* - Relation of aging and sex hormones to metabolic syndrome and cardiovascular disease. **Experimental Gerontology**. . ISSN 05315565. 46:7 (2011) 517–523. doi: 10.1016/j.exger.2011.02.007.

GUARNER, Veronica; RUBIO-RUIZ, Maria Esther - Metabolic Syndrome: Early Development and Aging. **Journal of Diabetes & Metabolism**. S2:01 (2011). doi: 10.4172/2155-6156.s2-002.

GUO, Shaodong - Insulin Signaling, Resistance, and the Metabolic Syndrome: Insights from Mouse Models to Disease Mechanisms Shaodong. **J Endocrinol.** . ISSN 15378276. 220:2 (2014) T1-Y23. doi: 10.1158/0008-5472.CAN-10-4002.BONE.

HAFFNER, Steven; TAEGTMEYER, Heinrich - Epidemic obesity and the metabolic syndrome. **Circulation**. . ISSN 00097322. 108:13 (2003) 1541–1545. doi: 10.1161/01.CIR.0000088845.17586.EC.

HAJDUCH, Eric; LITHERLAND, Gary J.; HUNDAL, Harinder S. - Protein kinase B (PKB/Akt) - A key regulator of glucose transport? **FEBS Letters**. . ISSN 00145793. 492:3 (2001) 199–203. doi: 10.1016/S0014-5793(01)02242-6.

HAN, Dong Ho *et al.* - Insulin resistance of muscle glucose transport in rats fed a high-fat diet: A reevaluation. **Diabetes**. . ISSN 00121797. 46:11 (1997) 1761–1767. doi: 10.2337/diab.46.11.1761.

HARRISON, Stephen A.; TORGERSON, Sigurd; HAYASHI, Paul H. - The natural history of nonalcoholic fatty liver disease: A clinical histopathological study. **American Journal of Gastroenterology**. . ISSN 00029270. 98:9 (2003) 2042–2047. doi: 10.1111/j.1572-0241.2003.07659.x.

HEINDEL, Jerrold J. *et al.* - Metabolism disrupting chemicals and metabolic disorders. **Reproductive Toxicology**. . ISSN 18731708. 68:2017) 3–33. doi: 10.1016/j.reprotox.2016.10.001.

HOLMAN, N.; YOUNG, B.; GADSBY, R. - Current prevalence of Type 1 and Type 2 diabetes in adults and children in the UK. **Diabetic Medicine**. . ISSN 14645491. 32:9 (2015) 1119–1120. doi: 10.1111/dme.12791.

HOSSAIN, Parvez; KAWAR, Bisher; NAHAS, Meguid EL - Obesity and diabetes in the developing world - A growing challenge. **New England Journal of Medicine**. . ISSN 15334406. 356:3 (2007) 213–215. doi: 10.1056/NEJMp068177.

HOTAMISLIGIL, Gökhan S. - Endoplasmic Reticulum Stress and the Inflammatory Basis of Metabolic Disease. **Cell**. . ISSN 00928674. 140:6 (2010) 900–917. doi: 10.1016/j.cell.2010.02.034.

HUGGETT, Robert J. *et al.* - Impact of Type 2 Diabetes Mellitus on Sympathetic Neural Mechanisms in Hypertension. **Circulation**. . ISSN 00097322. 108:25 (2003) 3097–3101. doi: 10.1161/01.CIR.0000103123.66264.FE.

INTERNATIONAL DIABETES FEDERATION - DIABETES ATLAS (8TH EDITION) - **(2017)** [Em linha] Disponível em WWW:<URL:http://diabetesatlas.org/resources/2017-atlas.html>.

ITURRIAGA, Rodrigo *et al.* - Carotid body chemoreceptors, sympathetic neural activation, and cardiometabolic disease. **Biological Research**. . ISSN 07176287. 49:1 (2016) 1–9. doi: 10.1186/s40659-016-0073-8.

ITURRIAGA, Rodrigo; ALCAYAGA, Julio - Neurotransmission in the carotid body: Transmitters and modulators between glomus cells and petrosal ganglion nerve terminals. **Brain Research Reviews**. . ISSN 01650173. 47:1–3 (2004) 46–53. doi: 10.1016/j.brainresrev.2004.05.007.

JACKSON, R. A. - Mechanisms of age-related glucose intolerance. **Diabetes Care**. . ISSN 01495992. 13:SUPPL. 2 (1990) 9–19.

JAYACHANDRAN, M. *et al.* - Status of lipids, lipid peroxidation, and antioxidant systems with vitamin C supplementation during aging in rats. **Journal of Nutritional Biochemistry**. . ISSN 09552863. 7:5 (1996) 270–275. doi: 10.1016/0955-2863(96)00027-7.

JUURINEN, Leena *et al.* - Effects of insulin therapy on liver fat content and hepatic insulin sensitivity in patients with type 2 diabetes. **American Journal of Physiology-Endocrinology and Metabolism**. . ISSN 0193-1849. 292:3 (2007) E829–E835. doi: 10.1152/ajpendo.00133.2006.

KAHN, Barbara B.; FLIER, Jeffrey S. - Obesity and insulin resistance Barbara. **The Journal of Clinical Investigation**. . ISSN 00970549. 106:4 (2000) 473–481. doi: 10.1007/BF01185522.

KAHN, C. R.; WHITE, M. F. - The insulin receptor and the molecular mechanism of insulin action. **Journal of Clinical Investigation**. . ISSN 00219738. 82:4 (1988) 1151–1156. doi: 10.1172/JCI113711.

KARIM, Sumera; ADAMS, David H.; LALOR, Patricia F. - Hepatic expression and cellular distribution of the glucose transporter family. **World Journal of Gastroenterology**. . ISSN 10079327. 18:46 (2012) 6771–6781. doi: 10.3748/wjg.v18.i46.6771.

KASHIWAGI, A. *et al.* - In vitro insulin resistance of human adipocytes isolated from subjects with noninsulin-dependent diabetes mellitus. **Journal of Clinical Investigation**. . ISSN 00219738. 72:4 (1983) 1246–1254. doi: 10.1172/JCI111080.

KASHYAP, Sangeeta *et al.* - A sustained increase in plasma free fatty acids impairs insulin secretion in nondiabetic subjects genetically predisposed to develop type 2 diabetes. **Diabetes**. . ISSN 00121797. 52:10 (2003) 2461–2474. doi: 10.2337/diabetes.52.10.2461.

KASHYAP, Sangeeta R.; DEFRONZO, Ralph A. - The insulin resistance syndrome: physiological considerations. **Diabetes and Vascular Disease Research**. . ISSN 1479-1641. 4:1 (2007) 13–19. doi: 10.3132/dvdr.2007.001.

KASSI, Eva *et al.* - Metabolic syndrome: Definitions and controversies. **BMC Medicine**. . ISSN 17417015. 9:48 (2011) 1–13. doi: 10.1186/1741-7015-9-48.

KATSIKI, Niki; MIKHAILIDIS, Dimitri P.; MANTZOROS, Christos S. - Non-alcoholic fatty liver disease and dyslipidemia: An update. **Metabolism: Clinical and Experimental**. . ISSN 15328600. 65:8 (2016) 1109–1123. doi: 10.1016/j.metabol.2016.05.003.

KAWASAKI, Takahiro *et al.* - Long-term sucrose-drinking causes increased body weight and glucose intolerance in normal male rats. **British Journal of Nutrition**. . ISSN 0007-1145. 93:5 (2005) 613–618. doi: 10.1079/bjn20051407.

KIM, Jong Yeon *et al.* - High-fat diet-induced muscle insulin resistance: Relationship to visceral fat mass. **American Journal of Physiology - Regulatory Integrative and Comparative Physiology**. . ISSN 03636119. 279:6 48-6 (2000) 2057–2065.

KINZIG, Kimberly P.; HONORS, Mary Ann; HARGRAVE, Sara L. - Insulin sensitivity and glucose tolerance are altered by maintenance on a ketogenic diet. **Endocrinology**. . ISSN 00137227. 151:7 (2010) 3105–3114. doi: 10.1210/en.2010-0175.

KLOP, Boudewijn; ELTE, Jan Willem F.; CABEZAS, Manuel Castro - Dyslipidemia in Obesity: Mechanisms and Potential Targets. **Nutrients**. . ISSN 20726643. 5:4 (2013) 1218–1240. doi: 10.3390/nu5041218.

KOBAYASHI, Daisuke *et al.* - Effect of pioglitazone on muscle sympathetic nerve activity in type 2 diabetes mellitus with alpha-glucosidase inhibitor. **Autonomic Neuroscience: Basic and Clinical**. . ISSN 15660702. 158:2010) 86–91. doi: 10.1016/j.autneu.2010.05.003.

KOTRONEN, Anna *et al.* - Liver fat in the metabolic syndrome. **Journal of Clinical Endocrinology and Metabolism**. . ISSN 0021972X. 92:9 (2007) 3490–3497. doi: 10.1210/jc.2007-0482.

KOTRONEN, Anna *et al.* - Liver Fat Is Increased in Type 2 Diabetic Patients and Underestimated by Serum Alanine Aminotransferase Compared With Equally Obese Nondiabetic Subjects. **Diabetes care**. 31:1 (2008) 165–169. doi: 10.2337/dc07-1463.Abbreviations.

KRAEGEN, E. W. *et al.* - In vivo insulin resistance in individual peripheral tissues of the high fat fed rat: assessment by euglycaemic clamp plus deoxyglucose administration. **Diabetologia**. 29:1986) 192–198.

KRISHNADATH, Ingrid S. K. *et al.* - Ethnic disparities in the prevalence of metabolic syndrome and its risk factors in the Suriname Health Study: A cross-sectional population study. **BMJ Open**. . ISSN 20446055. 6:12 (2016) 1–10. doi: 10.1136/bmjopen-2016-013183.

KROOK, Anna *et al.* - Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. **Diabetes**. . ISSN 00121797. 49:2 (2000) 284–292. doi: 10.2337/diabetes.49.2.284.

KUK, Jennifer L. *et al.* - Age-related changes in total and regional fat distribution. **Ageing Research Reviews**. . ISSN 15681637. 8:4 (2009) 339–348. doi: 10.1016/j.arr.2009.06.001.

KUMAR, Prem; PRABHAKAR, Nanduri - Sensing hypoxia: Carotid body mechanisms and reflexes in health and disease. **Respiratory Physiology and Neurobiology**. . ISSN 15699048. 157:1 (2007) 1–3. doi: 10.1016/j.resp.2007.02.003.

KYRIAKIS, John M.; AVRUCH, Joseph - Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. **Physiological Reviews**. . ISSN 00319333. 81:2 (2001) 807–869. doi: 10.1152/physrev.2001.81.2.807.

LAMBERT, Gavin W. *et al.* - Sympathetic nervous activation in obesity and the metabolic syndrome-Causes, consequences and therapeutic implications. **Pharmacology and Therapeutics**. . ISSN 01637258. 126:2 (2010) 159–172. doi: 10.1016/j.pharmthera.2010.02.002.

LAWAN, Ahmed; BENNETT, Anton M. - Mitogen-Activated Protein Kinase Regulation in Hepatic Metabolism. **Trends Endocrinol Metab.** 28:12 (2017) 868–876. doi: 10.1016/j.tem.2017.10.007.

LETURQUE, Armelle; BROT-LAROCHE, Edith; GALL, Maude LE - GLUT2 mutations, translocation, and receptor function in diet sugar managing. **American Journal of Physiology - Endocrinology and Metabolism**. . ISSN 01931849. 296:5 (2009) E985–E992,. doi: 10.1152/ajpendo.00004.2009.

LEWIS, Gary F. *et al.* - Effects of acute hyperinsulinemia on VLDL triglyceride and VLDL ApoB production in normal weight and obese individuals. **Diabetes**. . ISSN 00121797. 42:6 (1993) 833–842. doi: 10.2337/diab.42.6.833.

MAGNUSSON, I. *et al.* - Increased rate of gluconeogenesis in type II diabetes mellitus. **The Journal of clinical investigation**. . ISSN 0021-9738. 90:4 (1992) 1323–1327. doi: 10.1172/JCI115997.

MANCIA, Giuseppe *et al.* - 2007 Guidelines for the management of arterial hypertension. **European Heart Journal**. . ISSN 0195668X. 28:12 (2007) 1462–1536. doi: 10.1093/eurheartj/ehm236.

MANCIA, Giuseppe *et al.* - Impact of different definitions of the metabolic syndrome on the prevalence of organ damage, cardiometabolic risk and cardiovascular events. **Journal of Hypertension**. . ISSN 02636352. 28:5 (2010) 999–1006. doi: 10.1097/HJH.0b013e328337a9e3.

MANJUNATH, CN *et al.* - Atherogenic dyslipidemia. **Indian Journal of Endocrinology and Metabolism**. . ISSN 2230-8210. 17:6 (2013) 969–976. doi: 10.4103/2230-8210.122600.

MARQUES, Cláudia *et al.* - High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat. **Adipocyte**. . ISSN 2162397X. 5:1 (2016) 11–21. doi: 10.1080/21623945.2015.1061723.

MARSHALL, J. M. - Peripheral Chemoreceptors and Cardiovascular Regulation. **american physiological society**. 74:3 (1994) 543–594.

MARTINIS, Massimo DE *et al.* - Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity. **FEBS Letters**. . ISSN 00145793. 579:10 (2005) 2035–2039. doi: 10.1016/j.febslet.2005.02.055.

MCMURRY, MP *et al.* - Changes in lipid and lipoprotein levels and body weight in Tarahumara Indians after consumption of an affluent die. **The New England Journal of Medicine**. . ISSN 1476-069X. 325:24 (1991) 1704–1708. doi: 10.1186/1476-069X-12-14.

MELO, Bernardete F. *et al.* - Evaluating the Impact of Different Hypercaloric Diets on Weight Gain, Insulin Resistance, Glucose Intolerance, and its Comorbidities in Rats. **Nutrients**. 11:6 (2019) 1–19. doi: https://doi.org/10.3390/nu11061197.

MESOMYA, Wanpen *et al.* - Effect of Age on Serum Cholesterol and Triglyceride Levels in the Experimental Rats. **Nat. Sci.** 35:2001) 144–148.

MOLINA, Joseph M. *et al.* - Decreased activation rate of insulin-stimulated glucose transport in adipocytes from obese subjects. **Diabetes**. . ISSN 0012-1797. 38:8 (1989) 991–995. doi: 10.2337/diab.38.8.991.

MONZILLO, L. U. *et al.* - Effect of lifestyle modification on adipokine levels in obese subjects with insulin resistance. **Obesity research**. 11:9 (2003) 1048–1054.

MOORADIAN, Arshag D. *et al.* - Obesity-related changes in high-density lipoprotein metabolism. **Obesity**. . ISSN 19307381. 16:6 (2008) 1152–1160. doi: 10.1038/oby.2008.202.

MOORADIAN, Arshag D. - Dyslipidemia in type 2 diabetes mellitus. **NATURE CLINICAL PRACTICE ENDOCRINOLOGY & METABOLISM**. 5:3 (2009) 150–159. doi: 10.1038/ncpendmet.

MOORADIAN, Arshag D.; HAAS, Michael J.; WONG, Norman C. W. - Transcriptional Control of Apolipoprotein A-I Gene Expression in Diabetes. **Diabetes**. 53:5 (2004) 513–520.

MORLEY, John E. - Diabetes and Aging: Epidemiologic Overview. **Clinics in Geriatric Medicine**. . ISSN 07490690. 24:3 (2008) 395–405. doi: 10.1016/j.cger.2008.03.005.

NASCIMENTO, André F. *et al.* - A hypercaloric pellet-diet cycle induces obesity and co-morbidities in wistar rats. **Arquivos Brasileiros de Endocrinologia e Metabologia**. . ISSN 00042730. 52:6 (2008) 968–974.

NATIONAL CENTER FOR HEALTH STATISTICS, Division Of Health Interview Statistics - Crude and age-adjusted percentage of civilian, noninstitutionalized adults with diagnosed diabetes, United States, 1980–2010. **National Center for Chronic Disease Prevention and Health Promotion, Ed. Atlanta, GA, Centers for Disease Control and Prevention, Division of Diabetes Translation**. 2012).

NEUSCHWANDER-TETRI, Brent A.; CALDWELL, Stephen H. - Nonalcoholic steatohepatitis: Summary of an AASLD Single Topic Conference. **Hepatology**. . ISSN 02709139. 37:5 (2003) 1202–1219. doi: 10.1053/jhep.2003.50193.

NOLAN, Paul B. *et al.* - Prevalence of metabolic syndrome and metabolic syndrome components in young adults: A pooled analysis. **Preventive Medicine Reports**. . ISSN 22113355. 7:2017) 211–215. doi: 10.1016/j.pmedr.2017.07.004.

NOVAK, M. *et al.* - Gender differences in the prevalence of metabolic syndrome in 50-year-old Swedish men and women with hypertension born in 1953. **Journal of Human Hypertension**. . ISSN 09509240. 27:1 (2013) 56–61. doi: 10.1038/jhh.2011.106.

NUNN, Alistair V. W.; BELL, Jimmy D.; GUY, Geoffrey W. - Lifestyle-induced metabolic inflexibility and accelerated ageing syndrome: Insulin resistance, friend or foe? **Nutrition and Metabolism**. . ISSN 17437075. 6:2009) 1–26. doi: 10.1186/1743-7075-6-16.

PAN, Yang; PRATT, Charlotte A. - Metabolic Syndrome and Its Association with Diet and Physical Activity in US Adolescents. **Journal of the American Dietetic Association**. . ISSN 00028223. 108:2 (2008) 276–286. doi: 10.1016/j.jada.2007.10.049.

PARDAL, Ricardo *et al.* - Glia-like Stem Cells Sustain Physiologic Neurogenesis in the Adult Mammalian Carotid Body. **Cell**. . ISSN 00928674. 131:2 (2007) 364–377. doi: 10.1016/j.cell.2007.07.043.

PARDAL, Ricardo; LÓPEZ-BARNEO, José - Low glucose-sensing cells in the carotid body. **Nature Neuroscience**. . ISSN 10976256. 5:3 (2002) 197–198. doi: 10.1038/nn812.

PATON, Julian F. R. *et al.* - The carotid body as a therapeutic target for the treatment of sympathetically mediated diseases. **Hypertension**. . ISSN 0194911X. 61:1 (2013) 5–13. doi: 10.1161/HYPERTENSIONAHA.111.00064.

PESSIN, Jeffrey E.; SALTIEL, Alan R. - Signaling pathways in insulin action: Molecular targets of insulin resistance. **Journal of Clinical Investigation**. . ISSN 00219738. 106:2 (2000) 165–169. doi: 10.1172/JCI10582.

PISKURIC, Nikol A.; NURSE, Colin A. - Expanding role of ATP as a versatile messenger at carotid and aortic body chemoreceptors. **Journal of Physiology**. . ISSN 00223751. 591:2 (2013) 415–422. doi: 10.1113/jphysiol.2012.234377.

POKORSKI, M. *et al.* - The aging carotid body. **Journal of physiology and pharmacology : an official journal of the Polish Physiological Society**. . ISSN 08675910. 55 Suppl 3:1 (2004) 107–113.

PUGA, Guilherme M. *et al.* - Postprandial lipemia in the elderly involves increased incorporation of ingested fat in plasma free fatty acids and small (Sf 20-400) triglyceride-rich lipoproteins. **American Journal of Physiology - Endocrinology and Metabolism**. . ISSN 01931849. 301:2 (2011) 356–361. doi: 10.1152/ajpendo.00670.2010.

RAMLO-HALSTED, B. A.; EDELMAN, S. V. - The natural history of type 2 diabetes: Implications for clinical practice. **Primary Care - Clinics in Office Practice**. . ISSN 00954543. 26:4 (1999) 771–790. doi: 10.1016/S0095-4543(05)70130-5.

REAVEN, G. M. - Role of insulin resistance in human disease. **Diabetes**. . ISSN 00121797. 37:12 (1988) 1595–1607. doi: 10.2337/diab.37.12.1595.

REAVEN, Gerald M.; REAVEN, Eve P. - Age, Glucose Intolerance, and Non—Insulin-dependent Diabetes Mellitus. **Journal of the American Geriatrics Society**. . ISSN 15325415. 33:4 (1985) 286–290. doi: 10.1111/j.1532-5415.1985.tb07118.x.

REVIRIEGO, Jesús - Chapter 1 – Classification of Diabetes Mellitus: Criteria for Diagnosis. **Type 2 Diabetes Mellitus**. 2010) 1–23. doi: 10.1016/B978-84-8086-683-5.50001-9.

RIBEIRO, Maria J. *et al.* - Carotid body denervation prevents the development of insulin resistance and hypertension induced by hypercaloric diets. **Diabetes**. . ISSN 00121797. 62:8 (2013) 2905–2916. doi: 10.2337/db12-1463.

RIERA, Celine E.; DILLIN, Andrew - Tipping the metabolic scales towards increased longevity in mammals. **Nature Cell Biology**. . ISSN 14764679. 17:3 (2015) 196–203. doi: 10.1038/ncb3107.

RIVNER, Michael H.; SWIFT, Thomas R.; MALIK, Khalid - Influence of age and height on nerve conduction. **Muscle and Nerve**. . ISSN 0148639X. 24:9 (2001) 1134–1141. doi: 10.1002/mus.1124.

RODEN, Michael *et al.* - Mechanism of FFA-induced Insulin Resistance Rapid Publication Mechanism of Free Fatty Acid–induced Insulin Resistance in Humans. **J. Clin. Invest**. 97:12 (1996) 2859–2865.

RÖDER, Pia V *et al.* - Pancreatic regulation of glucose homeostasis. **Experimental and Molecular Medicine**. . ISSN 20926413. 48:2016). doi: 10.1038/emm.2016.6.

ROWE, John W.; TROEN, Bruce R. - Sympathetic nervous system and aging in man. **Endocrine Reviews**. . ISSN 0163769X. 1:2 (1980) 167–179. doi: 10.1210/edrv-1-2-167.

RYAN, Alice S. - Insulin resistance with aging: effects of diet and exercise. **Sports Medicine**. . ISSN 0112-1642. 30:5 (2000) 327–346.

SACRAMENTO, JF *et al.* - Adenosine Receptor Blockade by Caffeine Inhibits Carotid Sinus Nerve Chemosensory Activity in Chronic Intermittent Hypoxic Animals. **Advances in experimental medicine and biology**. 860:8 (2015) 133137. doi: 10.1007/978-3-319-18440-1_15.

SACRAMENTO, Joana F. *et al.* - Functional abolition of carotid body activity restores insulin action and glucose homeostasis in rats: key roles for visceral adipose tissue and the liver. **Diabetologia**. . ISSN 14320428. 60:1 (2017) 158–168. doi: 10.1007/s00125-016-4133-y.

SACRAMENTO, Joana F. *et al.* - Bioelectronic modulation of carotid sinus nerve activity in the rat: a potential therapeutic approach for type 2 diabetes. **Diabetologia**. . ISSN 1432-0428. 61:3 (2018) 700–710. doi: 10.1007/s00125-017-4533-7.

SACRAMENTO, Joana F. *et al.* - Contribution of adenosine and ATP to the carotid body chemosensory activity in ageing Joana. **J Physiol.** 2019). doi: 10.1113/JP274179.This.

SALTIEL, Alan R.; KAHN, C. Ronald - Insulin signalling and the regulation of glucose and lipid metabolism. **Nature**. . ISSN 1399-6576. 414:6865 (2001) 799–806. doi: 10.1111/j.1399-6576.2008.01868.x.

SANTULLI, Gaetano *et al.* - Age-related impairment in insulin release: The essential role of β 2-adrenergic receptor. **Diabetes**. . ISSN 00121797. 61:3 (2012) 692–701. doi: 10.2337/db11-1027.

SEPE, Anna *et al.* - Aging and regional differences in fat cell progenitors - A mini-review. **Gerontology**. . ISSN 0304324X. 57:1 (2011) 66–75. doi: 10.1159/000279755.

SEPPÄLÄ-LINDROOS, Anneli *et al.* - Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. **Journal of Clinical Endocrinology and Metabolism**. . ISSN 0021-972X. 87:7 (2002) 3023–3028. doi: 10.1210/jcem.87.7.8638.

SHANIK, Michael H. *et al.* - Insulin resistance and hyperinsulinemia: is hyperinsulinemia the cart or the horse? **Diabetes care**. . ISSN 19355548. 31 Suppl 2:2008) S262–S268. doi: 10.2337/dc08-s264.

SHAW, J. E.; SICREE, R. A.; ZIMMET, P. Z. - Global estimates of the prevalence of diabetes for 2010 and 2030. **Diabetes Research and Clinical Practice**. . ISSN 01688227. 87:3 (2010) 4–14. doi: 10.1016/j.diabres.2011.10.029.

SHEEDFAR, Fareeba *et al.* - Liver diseases and aging: Friends or foes? **Aging Cell**. . ISSN 14749718. 12:6 (2013) 950–954. doi: 10.1111/acel.12128.

SHIMOKATA, H. *et al.* - Age as independent determinant of glucose tolerance. **Diabetes**. . ISSN 00121797. 40:1 (1991) 44–51. doi: 10.2337/diab.40.1.44.

TABÁK, Adam G. *et al.* - Prediabetes : A high-risk state for developing diabetes. **Lancet**. 379:9833 (2012) 2279–2290. doi: 10.1016/S0140-6736(12)60283-9.Prediabetes.

TANG, X. *et al.* - Expression profile of mitrogen-activated protein kinase (MAPK) signaling genes in the skeletal muscle & liver of rat with type 2 diabetes: Role in disease pathology. **The Indian journal of medical research.** 2014).

TASKINEN, M. - Type 2 Diabetes as a Lipid Disorder. **Current Molecular Medicine**. . ISSN 15665240. 5:3 (2005) 297–308. doi: 10.2174/1566524053766086.

TASKINEN, M. R. - Diabetic dyslipidaemia: From basic research to clinical practice. **Diabetologia**. . ISSN 0012186X. 46:6 (2003) 733–749. doi: 10.1007/s00125-003-1111-y.

TENTOLOURIS, N.; LIATIS, S.; KATSILAMBROS, N. - Sympathetic system activity in obesity and metabolic syndrome. **Annals of the New York Academy of Sciences**. . ISSN 17496632. 1083:2006) 129–152. doi: 10.1196/annals.1367.010.

THORP, Alicia A.; SCHLAICH, Markus P. - Relevance of sympathetic nervous system activation in obesity and metabolic syndrome. **Journal of Diabetes Research**. . ISSN 23146753. 2015:2015) 1–11. doi: 10.1155/2015/341583.

TSIOUFIS, C. *et al.* - The incremental effect of obstructive sleep apnoea syndrome on arterial stiffness in newly diagnosed essential hypertensive subjects. **Journal of Hypertension**. 25:1 (2007) 141–146. doi: http://dx.doi.org/10.1097/HJH.0b013e32801092c1.

TSIOUFIS, Costas *et al.* - Pathophysiology of resistant hypertension: The role of sympathetic nervous system. **International Journal of Hypertension**. . ISSN 20900384. 2011:2011) 1–7. doi: 10.4061/2011/642416.

UCHIDA, K. *et al.* - Age-related changes in cholesterol and bile acid metabolism in rats. **Journal of Lipid Research**. . ISSN 00222275. 19:5 (1978) 544–552.

URANGA, Romina M.; KELLER, Jeffrey N. - Diet and age interactions with regards to cholesterol regulation and brain pathogenesis. **Current Gerontology and Geriatrics Research**. . ISSN 16877063. 2010:2010) 1–14. doi: 10.1155/2010/219683.

VANNI, Ester *et al.* - From the metabolic syndrome to NAFLD or vice versa? **Digestive and Liver Disease**. . ISSN 15908658. 42:5 (2010) 320–330. doi: 10.1016/j.dld.2010.01.016.

VAZQUEZ, Gabriela *et al.* - Comparison of body mass index, waist circumference, and waist/hip ratio in predicting incident diabetes: A meta-analysis. **Epidemiologic Reviews**. . ISSN 0193936X. 29:1 (2007) 115–128. doi: 10.1093/epirev/mxm008.

WANG, David Q. H. - Aging per se is an independent risk factor for cholesterol gallstone formation in gallstone susceptible mice. **Journal of Lipid Research**. . ISSN 00222275. 43:11 (2002) 1950–1959. doi: 10.1194/jlr.M200078-JLR200.

WANG, Hao; PENG, Dao Quan - New insights into the mechanism of low high-density lipoprotein cholesterol in obesity. **Lipids in Health and Disease**. . ISSN 1476511X. 10:1 (2011) 176–186. doi: 10.1186/1476-511X-10-176.

WANG, Yihong *et al.* - Glucagon-like peptide-1 can reverse the age-related decline in glucose tolerance in rats. **Journal of Clinical Investigation**. . ISSN 00219738. 99:12 (1997) 2883–2889. doi: 10.1172/JCI119482.

WESTERBACKA, Jukka *et al.* - Dietary fat content modifies liver fat in overweight nondiabetic subjects. **Journal of Clinical Endocrinology and Metabolism**. . ISSN 0021972X. 90:5 (2005) 2804–2809. doi: 10.1210/jc.2004-1983.

WINZELL, Maria Sorhede; AHRÉN, Bo - The High-Fat Diet-Fed Mouse: A Model for Studying Mechanisms and Treatment of Impaired Glucose Tolerance and Type 2 Diabetes. **Diabetes**. 53:3 (2004) 215–219.

WORLD HEALTH ORGANIZATION - World Health Organization: definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation. 2006).

WU, D. *et al.* - Aging Up-Regulates Expression of Inflammatory Mediators in Mouse Adipose Tissue. **The Journal of Immunology**. . ISSN 0022-1767. 179:7 (2007) 4829–4839. doi: 10.4049/jimmunol.179.7.4829.

YAN, Ming Xian *et al.* - Long-term high-fat diet induces pancreatic injuries via pancreatic microcirculatory disturbances and oxidative stress in rats with hyperlipidemia. **Biochemical and Biophysical Research Communications**. . ISSN 0006291X. 347:1 (2006) 192–199. doi: 10.1016/j.bbrc.2006.06.063.

YE, Jianping - Mechanisms of insulin resistance in obesity. **Frontiers of Medicine**. . ISSN 16737342. 7:1 (2013) 14–24. doi: 10.1007/s11684-013-0262-6.

YKI-JÄRVINEN, Hannele - Action of insulin on glucose metabolism in vivo. **Bailliere's clinical endocrinology and metabolism**. . ISSN 0950-351X. 7:4 (1993) 903–927.

ZAPATA, P.; STENSAAS, L.; EYZAGUIRRE, C. - AXON REGENERATION FOLLOWING A LESION OF THE CAROTID NERVE: ELECTROPHYSIOLOGICAL AND ULTRASTRUCTURAL OBSERVATIONS. **Brain Research**. 113:1976) 235–253. doi: https://doi.org/10.1016/0006-8993(76)90939-2.

ZHANG, Min; BUTTIGIEG, Josef; NURSE, Colin A. - Neurotransmitter mechanisms mediating low-glucose signalling in cocultures and fresh tissue slices of rat carotid body. **Journal of Physiology**. . ISSN 00223751. 578:3 (2007) 735–750. doi: 10.1113/jphysiol.2006.121871.

ZHAO, Feng-Qi; KEATING, Aileen F. - Functional Properties and Genomics of Glucose Transporters. **current Genimics**. 8:2007) 113–128.

ZIERATH, Juleen R. *et al.* - High-fat feeding impairs insulin-stimulated GLUT4 recruitment via an early insulin-signaling defect. **Diabetes**. . ISSN 00121797. 46:2 (1997) 215–223. doi: 10.2337/diab.46.2.215.