Miguel Carvalho Ravasco Milhano Correia

Licenciado em Bioquímica

Modulation of the carotid body activity to treat obesity

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

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

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Abstract

Nowadays the prevalence of obesity is rapidly increasing to levels higher than several decades ago. Associated to obesity are cardiovascular diseases, type II diabetes mellitus and cancer. Recently, it has been proposed that the carotid body (CB), a classic mediator of hypoxic responses, plays a role in energy and glucose homeostasis regulation and that its activity could be modulated to treat metabolic pathologies such as obesity and type II diabetes.

In this thesis, we have used a genetic animal model of obesity and diabetes, by the lack of functional leptin receptors, the Zucker diabetic fatty (ZDF) rats, to investigate the role of the CB in the development of obesity and to evaluate the impact of carotid sinus nerve (CSN) denervation on obesity and on metabolic dysfunction. Male ZDF rats with fasting glycemia over 120 mg/dl at 10 weeks old and lean controls were submitted to CSN resection or sham procedure (early stage group). Another group of animals was left until the 18^{th} week and submitted to CSN resection or sham procedure (late stage group). Weight gain, insulin sensitivity and glucose tolerance have been evaluated. Assessment of basal ventilation, as well as hypoxic and hypercapnic ventilatory responses, blood pressure, heart rate, and p_aO_2 was performed. After the sacrifice, the major fat depots' mass and liver lipid content was assessed.

In early and late stage animals, we found that CSN resection did not modify weight gain, glucose tolerance, fat mass deposition and insulin secretion. However, produced a small decrease in fasting glycemia, totally reversed insulin resistance and decreased lipid deposition in the liver.

We can conclude that modulation of the CB in this genetic model appears to play an important role in counteracting insulin resistance, however it does not seem to be a good method for the treatment of obesity *per se*.

Keywords: Obesity, insulin resistance, Zucker diabetic fatty rats, leptin, Carotid body, Carotid sinus nerve resection.

Resumo

Atualmente, a prevalência da obesidade está a aumentar rapidamente comparando a várias décadas atrás. Associadas à obesidade estão as doenças cardiovasculares, diabetes de tipo II e cancro. Recentemente, foi proposto que o corpo carotídeo (CB), um clássico mediador de respostas hipóxicas, participa na regulação homeostática energética e da glucose e a sua atividade pode ser modulada para tratar patologias metabólicas como a obesidade e a diabetes de tipo II.

Nesta tese, utilizámos um modelo genético animal da obesidade e diabetes, que não apresenta recetores de leptina funcionais, ratos *Zucker diabetic fatty* (ZDF), para investigar o papel dos CB no desenvolvimento da obesidade e avaliar o impacto da desnervação do nervo do seio carotídeo (CSN) na obesidade e disfunção metabólica. ZDF machos com glicemias em jejum de 120 mg/dl e controlos *lean* foram submetidos ao corte do CSN ou a um procedimento *sham* às 10 semanas (grupo estágio inicial). Outro grupo de animais foi apenas submetido ao corte do CSN ou ao *sham* às 18 semanas de idade (grupo estágio tardio). O ganho de peso, sensibilidade à insulina e tolerância à glucose foram avaliadas. A avaliação da ventilação basal, respostas ventilatórias à hipoxia e à hipercapnia, pressão arterial, frequência cardíaca e p_aO_2 também foi realizada. Depois do sacrifício, os maiores depósitos de gordura e o conteúdo lipídico do fígado também foram avaliados.

Nos animais dos estágios inicial e tardio, descobrimos que o corte do CSN não modificou o ganho de peso, tolerância à glucose, deposição de massa gorda e secreção de insulina. Porém, reduziu ligeiramente a glicemia em jejum, reverteu totalmente a resistência à insulina e diminuiu a deposição lipídica no fígado.

Podemos concluir que a modulação do CB neste modelo genético apenas parece contrariar a resistência à insulina, todavia não parece ser o melhor método para tratar a obesidade.

Palavras-chave: Obesidade, resistência à insulina, ratos *Zucker diabetic fatty*, leptina, corpo carotídeo, resseção do nervo do seio carotídeo.

Table of contents

Figures

Tables

Abbreviations

ADP – Adenosine diphosphate

Apo – Apolipoprotein

ATP – Adenosine triphosphate

AUC – Area under the curve

BAT – Brown adipose tissue

BMI – Body mass index

BP – Blood pressure

cAMP – cyclic-adenosine monophosphate

CB – Carotid body

CSN – Carotid sinus nerve

DBP – Diastolic blood pressure

FFA – Free fatty acid

FGF – Fibroblast growth factor

GLUT – Glucose transport protein

HDL – High-density lipoprotein

HR – Heart rate

IL – Interleukin

IR – Insulin receptor

IRS – Insulin receptor substrate

ITT – Insulin tolerance test

KITT – Constant rate for glucose disappearance

MBP – Mean blood pressure

NEFA - Non-esterified fatty acids

NPY – Neuropeptide Y

Ob-R – Leptin receptor

OGTT – Oral glucose tolerance test

PDE -Phosphodiesterase

PGC – Peroxisome proliferator-activated receptor gamma coactivator

PI3K – Phosphatidylinositol 3-kinase

PIP3 – Phosphatidylinositol 3,4,5-triphosphate

PRDI-BF – Positive regulatory domain I-binding factor

PRDM - PRDI-BF and RIZ homology domain containing protein

PPAR – peroxisome proliferator-activated receptor

R_f – Respiratory Frequency

RIZ – Retinoblastoma interacting zinc finger

SBP – Systolic blood pressure

SH2 – Src homology 2

SNS – Sympathetic nervous system

TAG – Triacylglycerol

TNF – Tumor necrosis factor

UCP – Uncoupling protein

V_E – Minute ventilation

VLDL – Very low-density lipoprotein

V_T – Tidal volume

WAT – White adipose tissue

ZDF – Zucker diabetic fatty

1. Introduction

1.1. Obesity as an epidemic disease: linking the disease with insulin resistance

Nowadays, all around the world, the prevalence of overweight people is much greater when compared to several decades ago. According to the World Health Organization, since 1980 the number of obese people has more than doubled, making this disease a public health problem which is spread indiscriminately across all ages, affecting more than 500 million adults (Cefalu *et al.*, 2015; Zafrir, 2013). The situation turns darker when, coupled to a higher body mass index (BMI) come several other pathologies that give birth to the metabolic syndrome, which has its central cause in abdominal obesity, and includes cardiovascular comorbidities, insulin resistance and type II diabetes, hypertension, obstructive sleep apnea disorder and even several kinds of cancer (Conde *et al.*, 2014; Goossens, 2008; Klop, Elte & Cabezas, 2013; McDonald *et al.*, 2011).

The Portuguese situation is not less serious, as for decades now there has been a scenario much like the one that is seen in the rest of the world. In 2014, the results of the enquiries made by Instituto Nacional de Estatística showed that more than half of

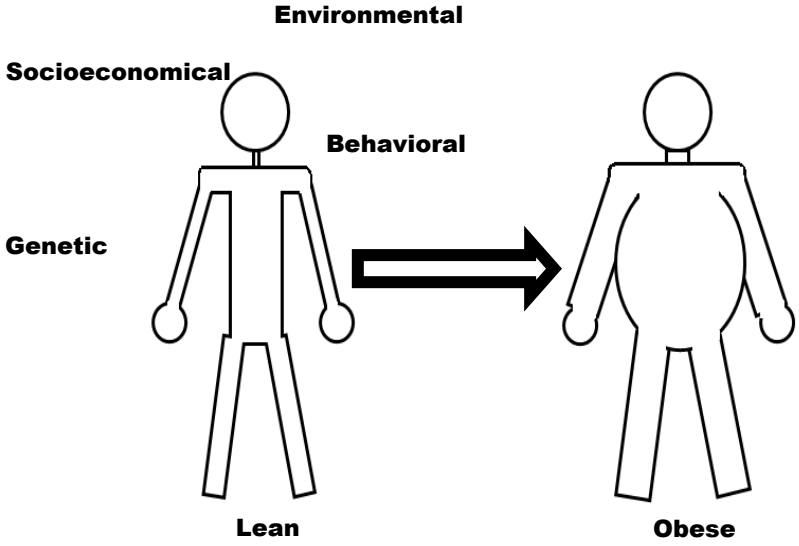

Figure 1 – Factors that (not only the now outdated, "lack of willpower" thinking) contribute to an increase in weight leading to obesity, development of cardiovascular problems, insulin resistance and diabetes.

the population aged 18 years old or older was overweight or obese (Instituto Nacional de Estatística, 2016; Santos, Kislaya & Gaio, 2016).

Obesity originates from different factors, not only environmental but also genetic, behavioral and socio-economical that culminate in a metabolic disorder which consists in the disturbance of the balance between energy uptake, energy expenditure and its storage (Figure 1) (Friedman & Halaas, 1998; Hausman *et al.*, 2001; Trayhurn, 2017). This balance becomes positive energetically and is followed closely by the increase in the size of the fat cells, adipocytes (Goossens, 2008; Trayhurn & Beattie, 2001; Zafrir, 2013). The excessive deposition of fats in the adipose tissue, responsible for the storage of energy in the form of fat molecules, is the main feature of this dysfunction. Consequently, associated with a hypertrophy of the adipose tissue, it begins an excessive accumulation of non-esterified fatty acids (NEFAs) and triacylglycerols (TAG) in other tissues like the liver, skeletal muscle and pancreas as well as in the blood, causing a pathology known as dyslipidemia. This, in turn, leads to a reduction in the sensitivity for insulin, due to the excess of energy in circulation, generating insulin resistance, characterized by the cells' inability to respond properly to this hormone (Boucher, Kleinridders & Kahn, 2014; Goossens, 2008). Studies using magnetic resonance spectroscopy with ^{13}C and ^{31}P have also discovered that, in diabetic patients, glycogenesis, the formation of glycogen, is impaired, resulting in a 50% reduction in the speed of glycogenesis comparing with control healthy patients (Bays *et al.*, 2013). Also, it has been found that insulin-activated glucose transport is affected in diabetic patients, further confirming a state of insulin resistance, mostly attributed to a lower expression and translocation of glucose transport protein (GLUT) 4.

In type II diabetes, the liver starts releasing glucose in an uncontrolled manner and this coupled with a lower rate for glycogenesis triggered by the peripheral insulin resistance, which in turn leads to the increase of insulin production by the β-cells in the pancreas originates hyperinsulinemia. In a later stage of the disease, this vicious cycle is closed when the pancreas is no longer able to cope with the increasing need of the organism for insulin, beginning its shutdown, and initiates a state of hypoinsulinemia with the subsequent increase in the glycemia values (hyperglycemia), worsening the disease. Obesity is a disease essentially metabolic in its nature and is hard to find isolated, since preceding it (or coming after it) is in most cases insulin resistance.

1.2. Glucose and lipids metabolism

Insulin action is essential for the normal glucose clearance. Mainly, this small protein (having only two polypeptide chains of 51 amino acids) works by increasing glucose uptake by cells, increasing glycogenesis in the liver and by inhibiting glucagon's secretion in the pancreas' α-cells (Balducci *et al.*, 2010). In insulin-sensitive tissues, such as the muscle, adipose tissue and liver, it initiates a kinase cascade by binding to insulin receptors (IR) at the cells' surface. IRs are tetrameric proteins with two extracellular subunits (α-subunits) that bind to the ligand and two intracellular subunits with tyrosine kinase activity (β-subunits). Upon insulin's binding, this receptor's β-subunits suffer a conformation change, promoting the auto-phosphorylation of its β-subunits. Afterwards, the receptor recruits substrates (commonly referred to as insulin receptor substrates, IRS) which bind to the β-subunits through pleckstrin and phosphotyrosine binding domains. In turn, these proteins are phosphorylated on several tyrosine residues originating a site for the binding of intracellular proteins with Src-homology 2 (SH2) domains (Boucher, Kleinridders e Kahn, 2014). These substrates call upon the phosphatidylinositol 3-kinase (PI3K) and integrate the serine-threonine kinase Akt 2 pathway. PI3K has a SH2 domain and binds to the phosphotyrosine-rich environment in the IRS, after which it phosphorylates the phosphatidylinositol 4,5-bisphosphate, creating phosphatidylinositol 3,4,5-trisphosphate (PIP3), a hydrophobic secondary messenger. This messenger then recruits Akt2 to the plasma membrane, which activates various processes involved in metabolism, growth and cell survival, to name a few (Boucher, Kleinridders & Kahn, 2014; Nelson *et al.*, 2008).

Akt2, once active, can induce glycogenesis by inactivating glycogen synthase kinase 3 and consequently activating glycogen synthase, which leads to an increase in glycogen in the liver. Also it can phosphorylate peroxisome proliferator-activated receptor gamma coactivator (PGC) 1-α, inhibiting gluconeogenesis, reducing the formation of glucose as well as the oxidation of fatty acids in the cells (Bays *et al.*, 2013; Boucher, Kleinridders & Kahn, 2014).

Another role for activated Akt2 is the regulation of lipolysis and β-cell insulin production. This is possible through the phosphorylation of phosphodiesterase (PDE) 3B, which becomes activated and decreases the concentration of cyclic-adenosine monophosphate (cAMP) in the cells. Furthermore, Akt2 is responsible for the deactivation of AS160, a protein that interacts with the vesicle transport-related Rab

proteins. This allows the increase in GLUT4 transport to the cell surface, increasing glucose uptake (Bays *et al.*, 2013; Boucher, Kleinridders & Kahn, 2014) (Figure 2).

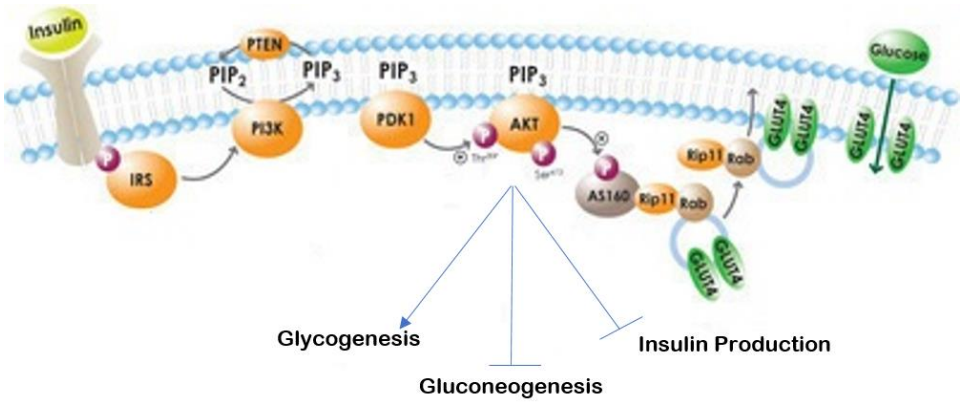

Figure 2 - Insulin pathway.
Upon its binding, insulin leads to the phosphorylation of its receptor and subsequently of the IRS. The latter will activate PI3K, which phosphorylate the phosphatidylinositol 4,5-bisphosphate into PIP3, a hydrophobic secondary messenger that travels through cell membrane and activates Akt. Akt can then have several outcomes, that ultimately lead to the control of glucose concentration in the blood (by turning it into glycogen, through glycogenesis, and by inhibiting gluconeogenesis) and generating a negative feedback loop, the production of insulin itself. Adapted from https://www.caymanchem.com/news/diabetes-and-insulin-signaling.

Lipids derived from fatty acids, triglycerides, follow a different route. When inside the organism, triglycerides are processed by lipases in the small intestine's lumen and broken down into free-fatty acids (FFA), monoacylglycerol, diacylglycerol and glycerol which are transported by passive diffusion or by specific transporters into the intestine's cells. Cholesterol is also transported into the intestine's cells (Klop, Elte & Cabezas, 2013; Nelson *et al.*, 2008). Triglycerides are then synthetized and cholesterol is changed into cholesterol-esters and then, along with the triglycerides and with the help of apolipoproteins (proteins that bind lipids, like apolipoprotein (Apo) B48) and phospholipids, they are all aggregated to originate chylomicrons, lipoproteic sphere particles. Depending on the composition of the chylomicrons these particles can be classified from very low density lipoproteins (VLDL) to high density lipoproteins (HDL), whether they are rich in lipids or rich in proteins, respectively. These particles are transported into the blood circulation, reaching the muscle or adipose tissues. If in the muscle, they will be recognized by lipoprotein lipases and hydrolyzed in β-oxidation

processes, to form energy, while in the adipose tissue they will be re-esterified into TAG, as large fat droplets, to store energy (Klop, Elte & Cabezas, 2013; Nelson *et al.*, 2008). The type of adipose tissue will ultimately determine the lipids' fate in the cell's metabolism.

1.3. Insulin production: indicator of insulin resistance

Insulin is one of the most important biomolecules for controlling the energy uptake in complex organisms like mammals. After a meal, insulin is produced by the β-cells in the pancreas' Langerhans islets as a physiological response to the increase in blood glucose levels. This hormone then works towards the stabilization of glucose levels by the mechanisms described previously in section 1.2. Since the insulin pathway is so complex, there are several steps where a malfunction can occur, both during its production and during its action at the cell level. These signaling malfunctions can also be originated in genetic malformations, these being rarer, causing defects in any member of the insulin signaling pathway from the IR to Akt. These can take place at the IR itself, more commonly so in cases of diet-induced insulin resistance (Boucher, Kleinridders & Kahn, 2014; Kahn & Flier, 2000).

Insulin is produced in the endoplasmic reticulum of β-cells as pre-proinsulin, being composed by three chains, A, B and C, the latter being a small connecting peptide, also known as C-peptide. When insulin is synthesized, the B chain (forming the proinsulin, with only the A and C chains) and later the C chains are cleaved from the A chain, in the liver. Then the C-peptide and insulin are secreted as a response to glucose intake into the organism. C-peptide is not known to have any biological functions; however, it is very useful as an indicator for endogenous insulin levels. Insulin is secreted from β-cells in response to glucose and travels to the liver where it suffers a process called insulin clearance, where C-peptide is cleaved from the A chain forming the functional insulin hormone (Ido *et al.*, 1997; Wilcox, 2005).

The release of insulin by the β-cells is controlled by several metabolic events that take place in the cytoplasm. The adenosine triphosphate (ATP)-sensitive K^+ channels normally allow the free flowing of potassium out of the cell. This process is mediated by the ATP/adenosine diphosphate (ADP) ratio inside the cell. When there is an increase in ATP caused by an increase in glucose uptake, these channels close, thus causing a depolarization of the cell's membrane, opening the Ca^{2+} channels on the cell's

surface. Ca^{2+} then acts as a secondary messenger responsible for the release of insulin held inside secretory granules found in the β-cells, thus leading to an increase in circulating insulin. In pathologies where insulin resistance is present there is also an impairment of glucose tolerance and the exocytotic mechanism of insulin, which is largely stimulated by the uptake of glucose, is greatly reduced. Therefore, a common treatment for increasing the secretion of insulin by the β-cells lies in the use of the sulfonylureas. The sulfonylureas can block the ATP-sensitive K^+ channels by binding to sulfonylurea receptors at the cell's surface, allowing for the increase in the insulin secretion without an increase in glucose uptake (Figure 3) (Alruwaili, 2016).

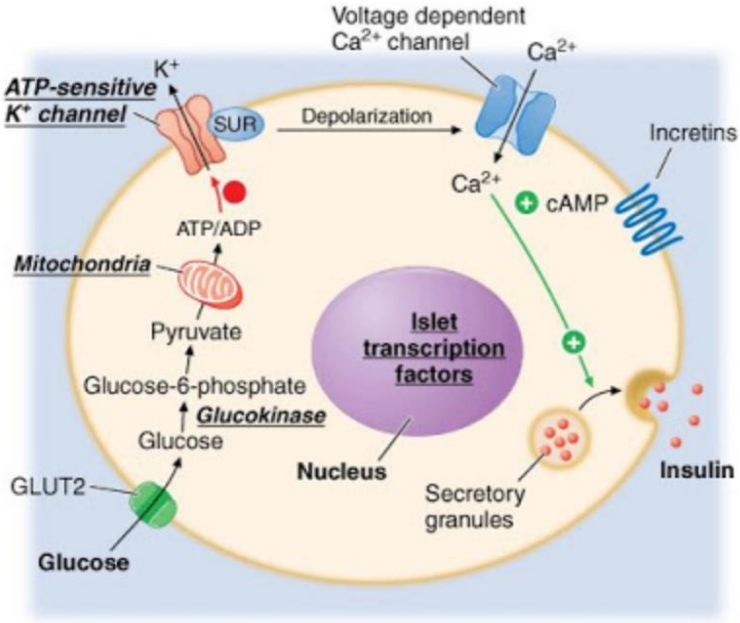

Figure 3 – Sulfonylurea-dependent insulin release.
Using sulfonylureas, insulin secretion by the pancreas' β-cells can be achieved by overriding the otherwise necessary glucose uptake and subsequent increase in ATP production. This allows for an increase in insulin secretion, allowing the treatment of hyperglycemic pathologies like type II diabetes and obesity. (Alruwaili, 2016).

Insulin production and its concentration in the blood are correlated with the level of peripheral insulin resistance. The greater the level of insulin concentration found in a pathological state like type II diabetes and/or obesity, the higher the insulin resistance will be (Wilcox, 2005). In insulin resistance states, hyperinsulinemia is very common because the pancreas keeps secreting insulin in order to achieve a normal glucose uptake. Obesity, for example, is a very common trigger for insulin resistance as shown by Figure 4, where β-cell function is altered through the years because of the peripheral

insulin resistance and overall impaired glucose tolerance. The general increase in adipose tissue hypertrophy found in obesity is also responsible for the onset of diabetes, characterized by the increases in glycemia and circulating FFA, thus leading to uncontrolled hyperglycemia.

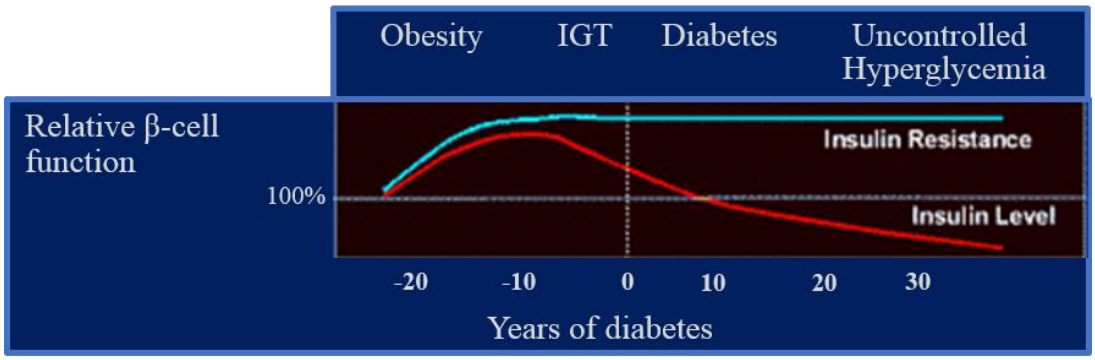

Figure 4 – Relative β-cell function over the progression of the disease.
From its beginning triggered by obesity, where the insulin resistance begins to take place in the periphery of the organism. This calls for an increase in β-cell activity due to an impaired glucose tolerance (IGT) followed by the development of type II diabetes. Later, the β-cell function begins to decline because of the reduced ability of the pancreas to deal with the uncontrolled hyperglycemia. Adapted from http://www.medscape.org/viewarticle/412860**.**

1.4. The adipose tissue as an endocrine and storage organ

Adipose tissue is the main lipid storage depot in humans and 3 types have been described: white, brown and beige. The white adipose tissue (WAT) that classically acts as a storage depot for energy which could be mobilized in times of need, releasing FFA to be oxidized for energy in other tissues, like the heart or the skeletal muscle (Klop, Elte & Cabezas, 2013; Trayhurn & Beattie, 2001); the brown adipose tissue (BAT), that is tasked to produce energy in the form of heat, in a process called non-shivering thermogenesis; and the beige adipocytes, that are a type of adipose tissue that is capable of producing heat through non-shivering thermogenesis like BAT, however it is found as a result of the transformation of certain WAT adipocytes derived from *myf5*-expressing precursors that have been exposed to stimuli like cold or adenylate cyclase activators, thus changing their phenotype (Figure 5) (Sidossis & Kajimura, 2015).

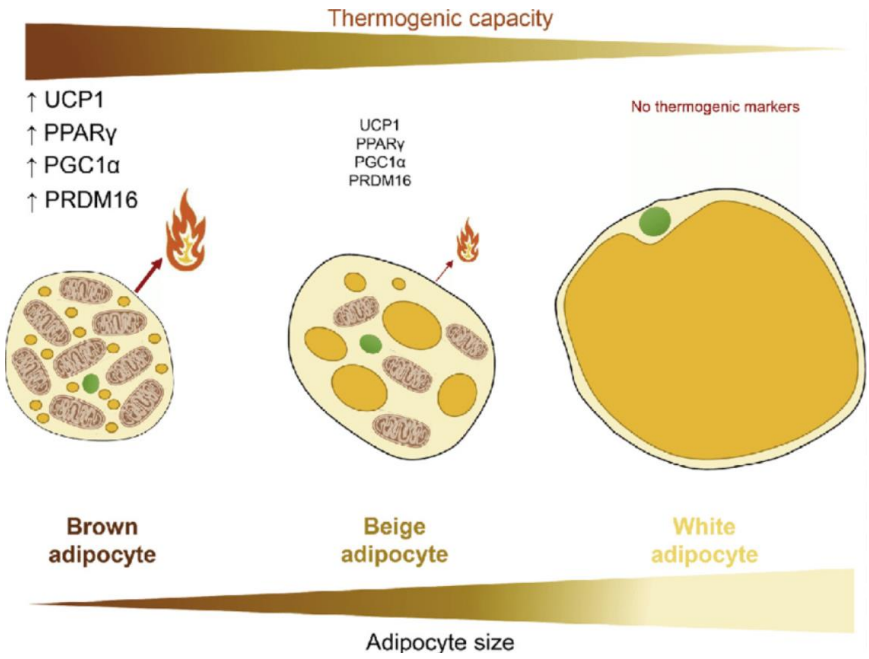

Figure 5 – Adipose tissue and its variety of phenotypes.
On one end of the spectrum, WAT is incapable of producing heat and does not express any thermogenic markers, like uncoupling protein (UCP) 1. Furthermore, it shows the biggest lipid droplets, often found as one single molecule and is a classic storage for energy. On the other end, BAT is the most thermogenically capable adipose tissue, expressing in very high levels thermogenic markers like UCP1, PGC1α, positive regulatory domain I-binding factor (PRDI-BF)1 and retinoblastoma interacting zinc finger (RIZ) homology domain containing (PRDM)16 and peroxisome proliferator-activated receptor (PPAR)γ. It has the highest mitochondrial content and the least lipid droplets, which it uses for generating heat. Between the two are the beige adipocytes, capable of producing some heat through non-shivering thermogenesis, although in a lesser extent than BAT adipocytes, but still higher than WAT adipocytes. It has several multilocular lipid droplets and intermediate mitochondrial content. (Contreras *et al.*, 2016).

1.4.1. White adipose tissue

WAT is made up by cells called adipocytes. These white adipocytes are big spherical cells (around 30-70 μm in diameter), filled almost entirely by one single fat droplet (TAG) which makes up for almost 65% of the cell's total volume. The nucleus and the few mitochondria are pushed against the cell's membrane, in the little space that is left (Nelson *et al.*, 2008). WAT depots are generally around the gonads, kidneys and subcutaneous and visceral regions (Qian, Huang & Tang, 2015). Its adipocytes are very active metabolically and respond very quickly to hormone stimuli, working in tandem with the liver, skeletal muscle and heart, but also have a connection with the central nervous system, as discussed in section 1.6 (Nelson *et al.*, 2008; Qian, Huang & Tang, 2015).

Adipocytes also have a central role in the production of adipokines, as a response to the presence of other hormones. These include: leptin, a hormone connected with the

regulation of appetite and with other physiological functions, including in breathing (Bassi *et al.*, 2016) and whose production was first discovered in the adipose tissue (for a review see: (Friedman & Halaas, 1998)); adiponectin, also known as adipocyte complement-related protein of 30 kDa, a versatile hormone that plays roles in energy metabolism, but also has anti-inflammatory and even anti-carcinogenic properties (for a review see (Nigro *et al.*, 2014)); and several mediators of inflammation as tumor necrosis factor (TNF)-α, interleukin (IL)-6, among other cytokines, and growth factors which act both systemically and locally, in tissues like the liver, muscles and brain and contribute to the maladaptive effects of obesity (for several reviews see: (Goossens, 2008; Qian, Huang & Tang, 2015; Trayhurn & Beattie, 2001)). It has also been demonstrated that adipose tissue has a close relationship with capillary networks. When the adipose tissue mass starts to expand, there is a need of oxygenation to the tissue and therefore there is a need to expand the capillary net. This is done in the presence of pro-angiogenic factors, which allow explants of subcutaneous tissue to form new blood vessels. Furthermore, a recent study by Min *et al*. from 2016 showed these vessels were able to differentiate into white adipocytes, first, in the presence of adipogenic factors, and then brown adipocytes, in the presence of browning factors (Min *et al.*, 2016). All this comes to show the untapped potential behind adipose tissue and its versatility.

1.4.2. Brown adipose tissue and UCP1

BAT, unlike WAT, shows a high mitochondrial content and several fat droplets scattered through the cell. This tissue arises as having a huge potential due to its ability to generate energy, which is dissipated in the form of heat. This energy has its origin in the uncoupling of the cellular respiration, which happens in the adipocytes' mitochondria's membranes.

The phenomenon of non-shivering thermogenesis occurs in BAT under basal conditions as the result of the expression of the mitochondrial protein UCP1 in the inner membrane of the mitochondria. This transmembrane protein acts as a proton channel, which, upon activation, uncouples the proton gradient generated during cellular respiration, releasing the chemical energy of this process in the form of heat. In the presence of cold, BAT can be stimulated by the sympathetic nervous system (SNS) and its transmitter, norepinephrine, activating the β3-adrenergic receptors at the cell's surface, activating lipolysis and mobilizing the fatty acids, which will be metabolized

during cellular respiration. The proton gradient, just like before, will be uncoupled and heat will be produced, through the presence of UCP1 (Almind *et al.*, 2007; Betz & Enerbäck, 2015; Lowell & Spiegelman, 2000).

BAT was found in a lesser extent in humans in comparison with other mammals, like rats, for example, which have a larger depot of this tissue in the interscapular region. Also, in humans, it has been found that UCP1-expressing tissue was inversely correlated with age and the increase in body weight (Harms & Seale, 2013). Aside from its evident role in the response to low temperatures by the increase in thermogenesis, BAT also plays an important part in the counteracting of infections during fever states, in the transition into wakefulness from hibernating mammals and also postnatally, to ensure the newly born can survive more easily the sudden differences in temperature (Betz & Enerbäck, 2015; Cannon & Nedergaard, 2004; Morrison & Madden, 2011).

1.4.3. Beige adipocytes

Curiously, under the effect of certain physical (for example, low temperatures) or chemical (adenylate cyclase activators, like forskolin or isoproterenol) stimuli, some WAT adipocytes can change their phenotype, giving rise to the so-called beige or brite (brown in white) cells. This change can be detected through the induction of the expression of genes commonly found in BAT, like *ucp1*, but also *tmem26* (cell surface marker for beige precursors) and the gene *pgc1α* (which stimulates mitochondrial biogenesis and the oxidative metabolism), for example (Betz & Enerbäck, 2015; Harms & Seale, 2013).

The factors involved in the formation of new beige adipocytes is not completely clear, although there are four theories that could explain its appearance in WAT depots. Either: they are derived from brown adipocyte precursors within WAT depots; or they appear as the result of the differentiation of an existing WAT adipocyte precursor; or they transdifferentiate from already mature WAT adipocyte into beige adipocytes; or they are derived from white adipocyte precursors expressing *myf5*, a smooth muscle cell marker (Stanford, Middelbeek & Goodyear, 2015).

It has been suggested that the induced formation of beige adipocytes from WAT could be used as a possible therapy to counter obesity. This could be achieved using drugs like rosiglitazone, a PPARγ agonist, that induce browning of the WAT adipocytes and lead to an improvement of the metabolic profile. Additionally, the induced

expression of hormones like irisin (an exercise-induced myokine that reverses diet-induced obesity and diabetes by stimulating BAT and beige's thermogenic activity) and fibroblast growth factor (FGF) 21 (a recently found adipokine that indicates non-shivering thermogenesis in humans) have been shown to be beneficial by stimulating BAT and beige activity. Furthermore, exercise is also believed to be a good therapeutic alternative to the use of drugs to promote beiging, not only because it increases the expression of irisin, but also because of its commonly known effects as a browning factor (Lee *et al.*, 2014; Nakhuda *et al.*, 2016).

In mice and rats, the browning of WAT as a result of exercise has been confirmed by several authors (Lee *et al.*, 2014; Stanford, Middelbeek & Goodyear, 2015), however, in humans there seems to be some controversy. A study by Nakhuda *et al* in 2016 in overweight and obese women submitted to a 16-week exercise program with modest calorie restriction showed an improvement in the metabolic profile and overall fitness, with fat mass loss, but did not show any increase in brown adipose tissue gene markers. In fact, in the subcutaneous region, it was observed a greater weight-loss that was associated with a lower expression of UCP1 and other brown molecular markers (Nakhuda *et al.*, 2016). All together this indicates that a suitable animal model that correctly resembles the human physiology is critical to understand the mechanisms underlying browning and the associated weight-loss due to exercise.

1.5. Leptin and its receptor, Ob-R

Although there is clear evidence in which states the SNS plays the most important role in controlling energy balance, for many years there was a clear lack of explanation for how energy balance was controlled, monitored and kept (Baak, van, 2001). That was until the discovery of leptin by Friedman *et al.* in 1994 (Zhang *et al.*, 1994). Leptin was first discovered by the positional cloning of its gene, the obese (*ob*) gene, on the mouse and the consequences of its mutations were soon identified to be in the genesis of the development of morbid obesity and type II diabetes (Friedman & Halaas, 1998; Zhang *et al.*, 1994). Later, the discovery of its receptor's gene, the diabetic (*db*) gene, shed even more light on the great importance of this hormone and its activity.

Leptin is expressed mainly in adipocytes and its expression correlates positively with the amount of adipose mass in the body. When leptin was discovered, it was first thought to be part of a negative-feedback loop to control the increase of adipose tissue

mass, relaying the information to the central nervous system, namely the hypothalamus (Friedman & Halaas, 1998). It works as ghrelin and neuropeptide Y (NPY)'s counterpart since it suppresses appetite and consequently energy intake, although this control of energy intake is a long-term effect and not an immediate effect of a meal. In spite of playing a clear role in energy balance and feeding behavior, leptin also has different functions on other physiological systems, like the immune system, for example, but also on the normal occurrence of puberty and even in bone formation (Friedman & Halaas, 1998; Takeda *et al.*, 2002). Leptin acts by binding to its receptor, the leptin receptor (Ob-R) on target cells, a member of the cytokine family of receptors. This receptor can have different isoforms and the one which allows the correct action of leptin is the Ob-Rb isoform, normally present in the hypothalamus but also other cell types. The activity of leptin on these hypothalamic receptors was further confirmed by the finding that intracerebroventricular injections of leptin greatly reduced food intake (Friedman & Halaas, 1998). The central effect of leptin on food intake was confirmed since the same dose had to be increased many times peripherally to achieve similar results, suggesting the hormone's ability to cross the blood brain barrier (Friedman & Halaas, 1998).

The lack of leptin (*ob/ob* genotype in mice; *fa/fa* in rats; both are autosomal recessive single point mutations) in the organism or its abnormal function (mutation on the receptor, *db*/*db* genotype), or the occurrence of the two simultaneously, can lead to different pathologies that range from severe obesity to a combination of diabetes and obesity (Coleman, 1978). Parabiotic experiments in mice have further demonstrated the importance of discerning the different phenotypes. The pairing of one mouse which is normal and another displaying a *db/db* phenotype, (with established obesity and diabetes caused by the lack of functional leptin receptors) will eventually lead to the normal mouse's death through starvation, due to the hyperleptinemic nature of the plasma of the mutant strain. On the other hand, the *ob/ob* mouse, which does not express leptin, when paired with a normal mouse can show improvements in the diseased mouse's insulin resistance and general obesity, thanks to the production of leptin by its peer (Coleman, 1978).

The Zucker Diabetic Fatty (ZDF) rat (Figure 6) was first found by Zucker and Zucker in 1961 when littermates started showing significant differences in shape and size, comparing to their normal counterparts, the symbol *fa* being chosen for the fat allele and *Fa* for the normal (Zucker & Zucker, 1961). The main feature of these rats is

the notable hyperphagia, more recently related with the mentioned lack of the action of leptin, which leads to obesity (Hempe *et al.*, 2012). This strain was later established as a genetic model for the study of diabetes, obesity and hypertension a couple of decades ago (Finegood *et al.*, 2001; Hempe *et al.*, 2012; Kurtz, Morris & Pershadsingh, 1989). In these rats, the *fa/fa* genotype induces the lack of functional leptin receptors, much in the same way of the *db/db* mice. Therefore, these rats display morbid obesity and hyperphagia. This animal model quickly develops insulin resistance, around six weeks of age. Afterwards, the onset of diabetes follows the already known sequence of events, starting with insulin resistance: hyperglycemia closely followed by hyperinsulinemia; failure of the pancreas' β-cells; hypoinsulinemia and finally the animal's inevitable death. Due to all these characteristics, it makes sense the use of this animal model for studying therapies and treatments for obesity, this century's epidemic. However, it should be mentioned that the phenotype presented by the ZDF rats is only seen rarely in obese humans, like most of the leptin-related deficiencies. These seem to appear with greater frequency (although this frequency is not very elevated) in consanguine populations (Clément *et al.*, 1998).

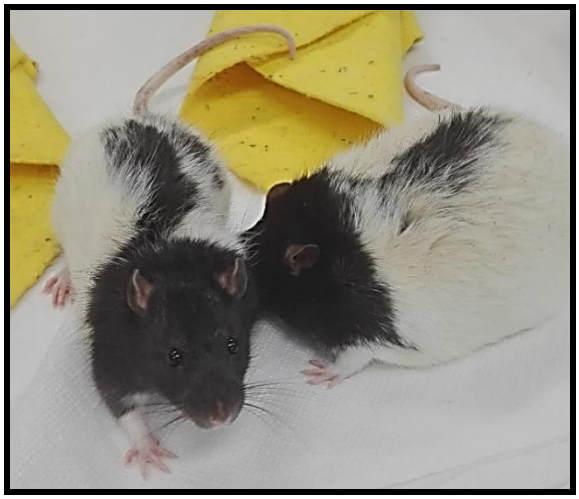

Figure 6 – The Zucker Diabetic Fatty Rat.
On the picture: left, the lean phenotype (Fa/?); right, the obese phenotype (Fa/Fa).

1.6. The sympathetic nervous system and the carotid body

The SNS is closely related to the regulation of energy balance in the organism, but mainly regulates its output, being influenced by several environmental and genetic

factors. Because of this, it is easy to understand its importance in the regulation of adipose tissue metabolism, both white and brown.

The SNS acts on the adipose tissue through the activation of its two receptors: α-adrenergic (α1 and α2) and β-adrenergic (β1, β2 and β3). By acting on these receptors, the SNS will have different effects. Upon stimulation of α_2-adrenergic receptors, lipolysis in the adipose tissue is inhibited, while when the β-adrenergic receptors are activated, lipolytic activity is increased (Baak, van, 2001).

Due to this SNS control of adipose tissue metabolism, it is expected dysfunction of adipose metabolism when SNS is altered. In fact, in obesity, where SNS activity is increased there is a substantial decrease in lipolysis, leading to the already mentioned hypertrophy of the adipocytes (Rayner, 2001). Also described is the link between SNS overactivation and insulin resistance in the peripheral tissues, including the skeletal muscle and adipose tissue (Ribeiro *et al.*, 2013). The uncontrolled activation of the SNS leads to an increase in lipolysis and to an increase in arterial blood pressure, leading to a reduction in glucose tolerance and resistance to insulin, as consequences from the increase in glucose and NEFAs in circulation (Conde *et al.*, 2014, 2016; Ribeiro *et al.*, 2013).

The carotid bodies (CBs) are peripheral chemoreceptors constituted mainly by two types of cells: the glomus cells, with chemoreceptor nature (type I) and the supporting glial-like cells (type II) (Kumar & Prabhakar, 2012). Type I cells are defined as being the chemoreceptor unit, and sense changes in the O_2, CO_2 and pH levels in the arterial blood. During hypoxia, hypercapnia or acidosis, the CBs are activated leading to an increase in the action potential frequency of their sensitive nerve, the carotid sinus nerve (CSN), which is integrated in the brainstem to produce a hyperventilation and to increase SNS activity. Aside from this, the CBs seem to directly activate the adrenal glands (Ribeiro *et al.*, 2013).

It has been previously demonstrated by Ribeiro *et al.* that the CBs are involved in glucose homeostasis and with peripheral insulin sensitivity. In fact, it was shown that CB dysfunction, observed as CBs overactivity, is associated with the development of insulin resistance and glucose tolerance, since the denervation of the CSN prevented and reversed insulin resistance and hypertension as well as glucose intolerance (Ribeiro *et al.*, 2013).

BAT, as mentioned before, expresses β_3-adrenergic receptors and is innervated both by sympathetic nerve fibers and sensory nerve fibers which makes this tissue, a target

and a mediator of SNS regulation (Almind *et al.*, 2007; Bartness, Vaughan & Song, 2010; Betz & Enerbäck, 2015; Lowell & Spiegelman, 2000). The binding of β_3-agonists to its receptors increases energy expenditure through lipolysis and fat oxidation. It is known that SNS is overactivated in obesity and metabolic diseases leading to increased levels of circulating catecholamines, like epinephrine or norepinephrine, which activate these receptors. Therefore, if there is an upset of the energy uptake/energy expenditure balance, like the one found in obesity, it would be expected the SNS interfered with BAT physiology, decreasing BAT activity. BAT can decrease in obesity its activity, through two pathways: one through the down regulation of β_3-adrenergic receptors or eventually through the dissipation of the proton motive force rises through the uncoupling of proton pumping and ATP synthase's activity, disturbing the normal synthesis of ATP and increasing the rate of non-shivering thermogenesis (Lowell & Spiegelman, 2000). In fact, it is known that in *ob/ob* mice, the SNS output to BAT is greatly reduced. The CB has been suggested to be one of the key triggers in SNS activity driving insulin-resistance, and it was shown that the CSN resection in metabolic syndrome animals normalizes the sympathetic activity (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017). Therefore, if SNS controls BAT there would be an expected increase in BAT mass and function, subsequently leading to fat mass loss and overall weight loss through heat production in CSN-resected animals.

All these findings support the hypothesis that the CBs have a central role in mediating energy expenditure through the SNS's activity (Figure 7). As such, the link between the CB and the SNS must be investigated further to better understand its mechanism in glucose metabolism and insulin resistance. Additionally, the link between CB, SNS and BAT should be clarified especially in the context of obesity and metabolic disturbances.

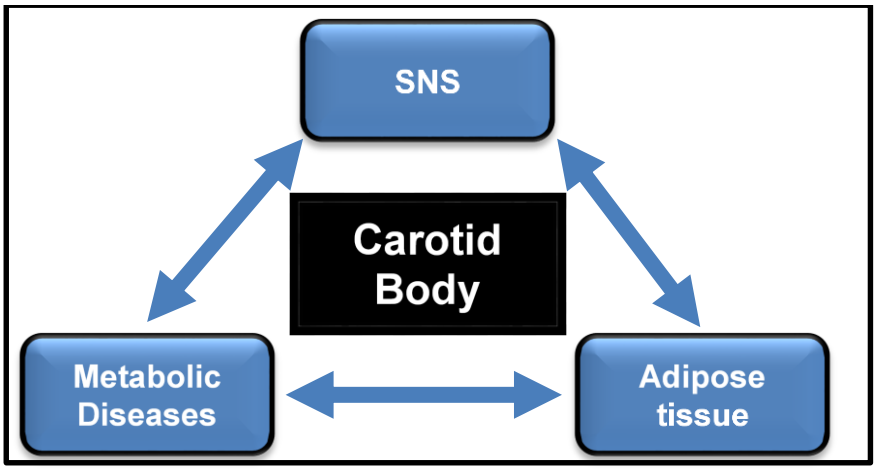

Figure 7 - Possible link between the carotid body, the SNS, metabolic diseases and the adipose tissue.

2. Aims

Obesity has been established as the 21st century epidemic, being the most important cause of cardiovascular and metabolic disturbances and contributing to significant morbidity and mortality worldwide. Therefore, the investigation of the pathophysiological mechanisms behind the development of obesity and obesity-related diseases urge in order to find new therapeutic targets to treat this epidemic.

The general aim of this thesis was to investigate the role of the CB in the development of obesity and to evaluate the impact of CSN denervation on obesity and on metabolic dysfunction in a genetic obese animal model, the ZDF rat.

The specific aims of this thesis were:

1 – Evaluate the impact of CSN denervation on weight gain, fat mass, lipid deposition and lipid profile in an early and late stage of obesity and metabolic dysfunction;

2 – Evaluate the impact of CSN denervation on insulin sensitivity, glucose tolerance and insulin and C-peptide levels in an early and late stage of obesity and metabolic dysfunction;

3 – Evaluate the impact of CSN denervation on basal ventilation and on the ventilatory responses to hypoxia and hypercapnia in an early and late stage of obesity and metabolic dysfunction;

4 – Evaluate the impact of CSN denervation on cardiovascular/hemodynamic parameters in an early and late stage of obesity and metabolic dysfunction.

3. Methods

3.1. Animal care and diets

Experiments have been performed using 2 groups of male animals: the ZDF (Fa/Fa) rats, as a model of obesity and metabolic disease and Zucker Lean (Fa/?) rats as controls. Animals were ordered from Charles River Laboratories (Paris, France) with six weeks of age and have been maintained during 2 weeks in quarantine. Upon arrival animals were divided and kept in groups of two (ZDF rats) and three (Lean) rats per cage, and housed in an environmentally controlled space (21±ºC temperature; 55±10% humidity) with 12 h light/dark cycles. ZDF animals have been fed with Purina 5008 (Formulab Diet 5008 and Formulab Diet 5008C33, Purina) and consisted of a mix of 23.6% protein, 14.8% lipid, 50.3% carbohydrates, 3.3% fiber and the remaining minerals and vitamins. The Lean animals fed a control diet (7.4% lipid and 75% carbohydrates, of which 4% were sugars and 17% protein; SDS diet RM1). The total energy provided by the different groups of nutrients was for the ZDF animals 4.36 kcal/g of gross energy, of which 3.56 have physiological fuel value. The Zucker lean's diet had 3.5 kcal/g of gross energy of which 2.84 have physiological value (the diets' caloric values were determined from the diet sheets found for the diets on the websites of the respective producing companies).

3.2. Experimental design

To evaluate the role of the CB and the impact of CSN denervation in different stages of disease development, we have divided the groups in 2: an early stage group in which the animals had 10-11 weeks of age when they were submitted to chronic CSN denervation, and a late stage group in which the animals were submitted to a chronic CSN denervation at the 18th week of age. Sham procedures were done to control groups. During all the experimental period, body weight, food and water intake and metabolic parameters, as fasting glucose, insulin sensitivity and glucose tolerance have been monitored. Additionally, respiratory parameters, as respiratory frequency and tidal volume have been evaluated by whole-body plethysmography until the end of the experimental protocol (Figure 8).

After denervation, the animals have been evaluated for metabolic parameters each 1-2 weeks to evaluate the impact of CSN denervation. For the early stage group, the

animals have been monitored during 7 weeks post-resection and for the late stage group animals have been analyzed for 3 weeks post-resection.

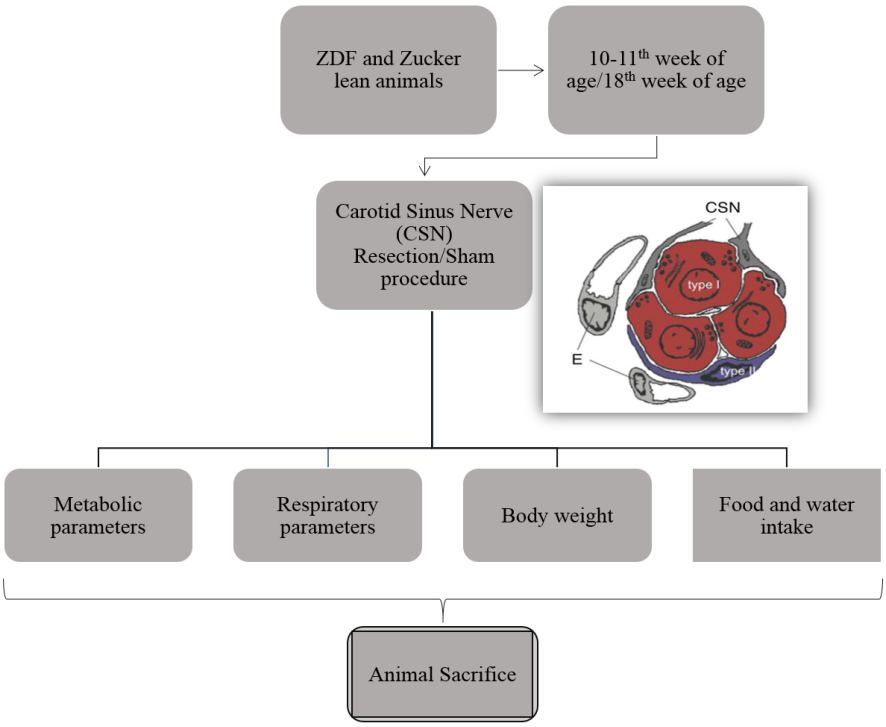

Figure 8 - Workflow for this project.
CB image was adapted from Chang J., Andy *et al*., Nature, 2015**.**

3.3. Surgical and animal sacrifice procedures

The surgical procedure was done after anesthetizing the animals with a mixture of ketamine (30 mg/kg)/medetomidine (4 mg/kg). Carprofen (5 mg/kg) was administered as an anti-inflammatory immediately before the surgical procedure and for more 3 days following the procedure. After surgery, the animals were sutured and received atipamezole (2 mg/kg), to counter the anesthetic effect. Buprenorphine (10 µg/kg) was administered when animals were already totally awaked.

For the terminal experiment, animals have been anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and the femoral artery was catheterized to measure the arterial blood pressure (systolic (SBP), diastolic (DBP) and mean (MBP) blood pressure and heart rate (HR), using a blood pressure transducer (-50, +300 mmHg) from EMKA Technologies (Paris, France). Also, p_aO_2 was measured using a capnograph (from EMKA Technologies (Paris, France)). Afterwards, a cardiac puncture was performed to collect samples of plasma to EDTA-containing tubes and serum. Tissues as the liver, soleus and gastrocnemius, diaphragm, visceral, perinephric, epididymal, subcutaneous

and brown adipose tissues (collected from the interscapular region), brain and hypothalamus samples were then rapidly collected and stored at -80 ºC, for further analysis by Western Blot and other techniques described further down below (Ribeiro *et al.*, 2013). Animal care followed the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU). Protocols were approved by the ethics committee of the Faculty of Medical Sciences (Ribeiro *et al.*, 2013).

3.4. Evaluation of the insulin sensitivity

The insulin sensitivity was determined through an insulin tolerance test (ITT). For that, the animals were submitted to 16 h fasting with free access to water in the previous night. After the fasting period and immediately in the morning, the basal plasma glycemia levels were measured and a bolus of insulin (0.1 U/kg) was administrated in the tail vein (Monzillo & Hamdy, 2003; Sacramento *et al.*, 2015). The plasma glycemia levels were then measured every minute during 15 minutes. 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 (KITT) was used to evaluate the insulin sensitivity, and when the KITT is >3.5 means that the animals are insulin sensitive and when the KITT is <3.5 the animals are insensitive to insulin action. The constant is obtained from the equation:

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

Glucose half-time was measured from the slope of the curve during the linear phase of disappearance of glucose (Monzillo & Hamdy, 2003; Sacramento *et al.*, 2015). These tests were performed to evaluate the animals' insulin sensitivity before the surgical procedure and on the 1st, 2nd, 3rd, 5th and 7th week after surgery, in the early stage animals, and before, 1st, 2nd and 3rd weeks on the late stage animals.

3.5. Evaluation of the glucose tolerance

Glucose tolerance was evaluated through an oral glucose tolerance test (OGTT). This test was performed to determine the glucose tolerance and subsequently the speed of release of insulin and its action in the peripheral tissues, after an overnight fast. It

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

At the end of the OGTT, blood serum samples were collected from a small cut made on the tail vein, for biomarker evaluation (Torres-Villalobos *et al.*, 2016). OGTTs have been done at the same time points as the ITTs.

3.6. Recording of basal ventilation and ventilatory responses to hypoxia and hypercapnia through whole-body plethysmography

Every two weeks, ventilation was measured in conscious freely moving rats by whole-body plethysmography for the determination of ventilation parameters (respiratory frequency, R_f, in breaths per minute; tidal volume (V_T), in milliliters). From the product of these parameters, the minute ventilation of the animals was obtained (V_E, in milliliters per minute per kilogram) (Hernandez *et al.*, 2012). The system (EMKA Technologies, Paris, France) consisted of 5-litre methacrylate chambers continuously fluxed (2 l/min) with gases.

Each rat was placed in the plethysmography chamber and allowed to breathe room air for 30 min to allow adaptation to chamber environment and to acquire a standard resting behavior. After acclimatization period the protocol consisted in 10 minutes of normoxia (21% O_2), followed by 10 minutes of hypoxia (10% O_2), 10 minutes of normoxia, followed by 10 minutes of hypercapnia (5% CO_2) and finally 10 minutes of normoxia. The gases used were balanced by N_2. R_f and V_T levels obtained when animals where moving where excluded. Each plethysmography chamber was calibrated by the injection of a volume of 20 ml of air with a syringe into each of the chambers (Conde *et al.*, 2012). The software used for the visualization and analysis of the data was IOX 2.9.5.73 from EMKA Technologies, Paris, France.

3.7. ELISA kits for the quantification of insulin and C-peptide in blood serum samples.

Commercial ELISA kits were used for the determination of insulin (Mercodia Ultrasensitive Rat Insulin ELISA, Uppsala, Sweden) and C-peptide (Mercodia Rat C-

peptide ELISA, Uppsala, Sweden) in serum blood samples. The protocol used was that described on the pamphlet found inside the kit.

3.8. Quantification of the lipid content in the liver

A protocol modified from the protocol described by Elena Olea Fraile (Fraile, 2015) was used for the determination of the amount of lipids in the liver. This protocol consisted in the homogenization of a small amount of tissue using Folch's reagent (chloroform: methanol, 2:1) (Folch, Lees & Stanley, 1957). For pulverizing the tissue, it was used a Bessman tissue pulverizer of medium size from Spectrum Laboratories, with liquid nitrogen. After the homogenization, the tissues were collected to glass tubes and were submitted to agitation during 2 hours, using an automatic tube shaker. After agitation, the solution was filtered with filter paper into another glass tube. In the new glass tube, another 2.5 ml of Folch's reagent were added to the remaining tissue. The agitation and filtration, followed by new addition of the Folch reagent were repeated twice. After the third and final filtration, it was added a NaCl 0.73% solution, after which it was stirred vigorously and left to rest overnight. In the ensuing day, the two resulting phases were separated and the organic (upper) phase was collected in a Petri dish previously weighted. To the phase remaining in the tube it was added a solution of Folch's reagent: NaCl 0.53% (80:20). This new mix was stirred again and left resting for another 2 hours. As earlier, the organic phase resulting from this last mix with the Folch's reagent was separated from the aqueous phase and added to the Petri dishes. After the complete drying of the dishes, they were weighted again and the percentage of lipids in the tissue was determined, according to the initial weight used in the experiment (Fraile, 2015).

3.9. Data Analysis

Data was evaluated in a 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 $P<0,05$ (Kobayashi e Pillai, 2013). The group considered as control in all tests and evaluations was the lean sham group.

4. Results

4.1. Caloric and liquid intake is not affected by the CSN resection nor the surgery

Animals were randomly allocated to CSN resection or sham surgery groups, in which the animals have been submitted to the same surgical procedure but the CSN was left intact. CSN bilateral resection or sham procedure did not modify significantly animal behavior nor caloric intake, measured as the average caloric intake per day during the 7 weeks after CSN denervation, on the early stage animals, and 3 weeks after the CSN denervation, on the late stage animals. Liquid intake was also measured as the average liquid intake per day, for the same periods.

Between the Fa/Fa and lean animals, a big difference is visible both in the caloric and liquid intakes, even before submitting the animals to the surgical procedure. This happened both in the early and late stage groups. The intakes measured for the two different animal groups were significantly different being 41% and 38% higher, for the caloric and liquid intake, respectively, in the early stage group (Table 1); and 71% and 111% higher, for the caloric and liquid intake, respectively, in the late stage group (Table 2).

Also between the Fa/Fa groups before the surgery of the early stage we found there was a significant difference of 34% in the liquid intake. This difference was dissipated after the surgery.

The liquid intake measured for the Fa/Fa animals in the early stage group did not change significantly after the surgery, when comparing to the lean animals, remaining at a 41% higher value. The caloric intake, on the other hand went down to a value 25% higher (Table 1).

The late stage group showed the biggest changes when comparing lean and Fa/Fa animals after the surgery, decreasing the differences to 54% and 75%, respectively for the caloric and liquid intakes (Table 2).

Between the sham and denervated animals, both in the early and late stage groups, no significant differences were found, when comparing the values for the caloric and liquid intakes. (Tables 1 and 2)

Table 1 - Early stage caloric and liquid intake, before and after surgery.

	Caloric Intake (kcal/day/kg)		Liquid Intake (ml/day/kg)	
	Before surgery	After surgery	Before surgery	After surgery
Lean Sham (n=6)	294.50 ±6.942	210.71 ± 4.999	119.47 ± 1.714	85.57 ± 3.215
Lean Denervated (n=6)	292.76 ± 3.952	221.52 ± 5.082	119.12 ± 1.682	88.26 ± 2.550
Fa/Fa Sham (n=5)	417.76 ± 13.749****	298.91 ± 14.464****	143.13 ± 8.759***	126.95 ± 8.348*
Fa/Fa Denervated (n=4)	411.14 ± 15.804****	262.60 ± 6.038**	192.68 ± 31.618***#	120.86 ± 6.491

Values are displayed as the means of all the animals' caloric and liquid intake values with their SEM values. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 vs lean sham; #P<0.05 vs Fa/Fa sham (Two-way ANOVA Bonferroni test).

Table 2 – Late stage caloric and liquid intake. before and after surgery.

	Caloric Intake (kcal/day/kg)		Liquid Intake (ml/day/kg)	
	Before surgery	After surgery	Before surgery	After surgery
Lean Sham (n=1)	193.26	184.15	80.14	79.25
Lean Denervated (n=1)	193.26	178.26	80.14	107.30
Fa/Fa Sham (n=2)	329.71 ± 17.020	259.95 ± 38.269	154.72 ± 27.418	163.53 ± 63.433
Fa/Fa Denervated (n=3)	332.50 ± 10.215	293.65 ± 2.457	178.858 ± 28.863	163.45 ± 11.679

Values are displayed as the means of all the animals' caloric and liquid intake with their SEM values.

4.1.1. Effect of Fa/Fa phenotype and of CSN resection on weight gain

As previously described in section 3.2, the early stage group has been submitted to denervation between weeks 10 and 11. The growth rates were determined from the weekly increase in weight, before and after the surgery, in g per week (Table 3).

Figure 9 shows a steady growth in both lean and Fa/Fa animals, although the Fa/Fa rats quickly outgrow their lean counterparts, which grow at a less visible rate, increasing the difference from 37% in the first week (Lean = 140.7 ± 4.55 g; Fa/Fa = 179.8 ± 6,31 g) to 49% in the last weeks, even after the denervation (Lean = 328.7 ± 6.63 g; Fa/Fa = 487.1 ± 9.06 g).

As of the day of the surgery, the lean denervated group's average weight drops slightly and this difference carries out for the remainder of the experiment. This difference reaches 11.7% on the first week and 11.6% on the second week after surgery and is significantly different on the 1st, 2nd, 3rd and 5th weeks following surgery (Lean sham 1 week after surgery = 244.0 ± 9.31 g; Lean sham 2 weeks after surgery = 258.0 ±

10.42 g; Lean denervated 1 week after surgery = 215.3 ± 5.77 g; Lean denervated 2 weeks after surgery = 228.0 ± 6.15 g).

The Fa/Fa animals don't show a very clear difference between the two groups, not reaching more than 5% on the 2nd week after the surgery (Fa/Fa sham = 364.4 ± 8.13 g; Fa/Fa denervated = 343.5 ± 9.60 g). The growth rate of all animals determined after the surgeries displays a considerable drop, comparing to those before the surgeries. This change, however, is not significant.

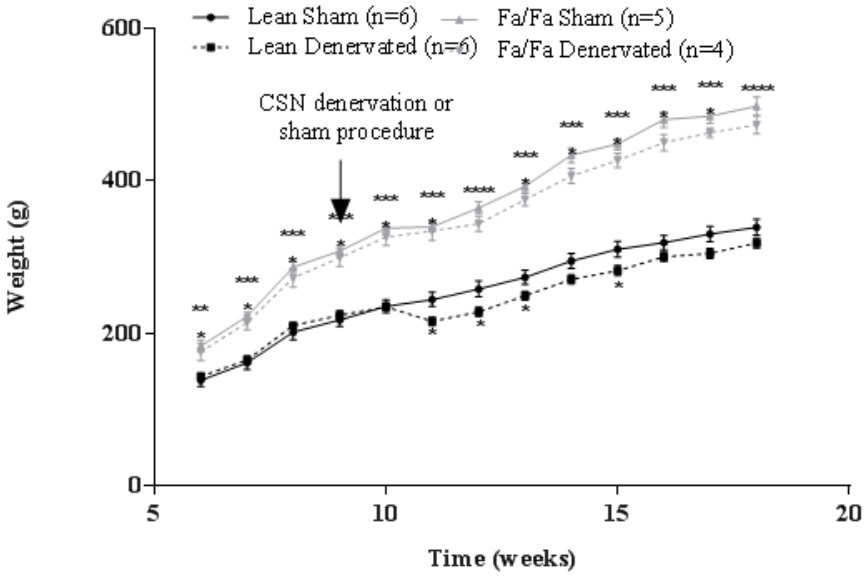

Figure 9 – **Early stage animal growth curves.**
Near the 10th week of diet. some of the animals were selected on both groups for the CSN resection, forming the denervated groups. This point is highlighted by the black arrow. ***P<0.001; ****P<0.0001 vs lean sham group (Two-way ANOVA Bonferroni test).

Table 3 – **Growth rates for the early stage animals.**

	Before Surgery (g/week)	**After Surgery (g/week)**
Lean Sham (n=6)	24.20 ± 5.353	12.96 ±1.568
Lean Denervated (n=6)	22.83 ± 7.813	10.46 ± 4.705
Fa/Fa Sham (n=5)	38.60 ± 9.444	20.05 ± 4.838
Fa/Fa Denervated (n=4)	37.63 ± 7.49	18.44 ± 3.402

The growth rate was assessed by calculating the amount of weight gained by the different animals, per week. Data is shown as the mean and SEM.

The late stage group was maintained in the same conditions as the early stage group. but for a longer period. Between the 18th and 19th week of diet, the animals were

randomly selected for the CSN resection or the sham procedure. The different groups' growth curves are represented in Figure 10. In addition, the growth rates were determined from the weekly gain of weight, every week, before and after the surgery, accordingly to the early stage animals. As with the early stage group, the animals in both groups were weighed weekly, on the weekday of the surgery (Table 4).

The figure shows that there is no significant difference between the lean groups, there being no visible change upon the CSN resection of the denervated animals. This difference remains steady around 10% throughout the test. On the other hand, the Fa/Fa groups become interestingly distinct after the surgeries where the denervated group's average weight drops by 8% on the 1st week after surgeries differentiating it from the sham group's average (Fa/Fa sham = 466.0 ± 48.00 g; Fa/Fa denervated = 426.7 ± 1.76 g). This change however is not significant. Two weeks after the surgery, the Fa/Fa denervated animals' weight was 6% lower than the Fa/Fa sham and one week after this, 5% (Fa/Fa sham 2 weeks after surgery = 470.0 ± 50.00 g; Fa/Fa sham 3 weeks after surgery = 463.0 ± 47.00 g; Fa/Fa denervated 2 weeks after surgery = 442.0 ± 2.00 g; Fa/Fa denervated 3 weeks after surgery = 438.0 ± 5.03 g). After the surgery, a big difference in the growth rate is evident in all groups, which decreases substantially, from what is seen on Table 4.

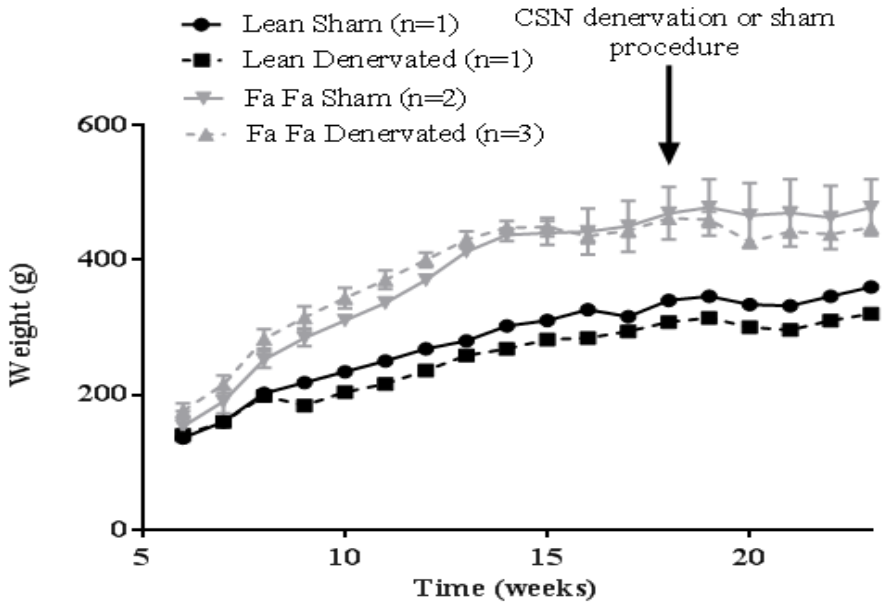

Figure 10 – Late stage animals' growth curves.
The black arrow shows the point in which the animals were denervated or kept as sham controls, for both groups.

Table 4 – **Growth rates for the late stage animals.**

	Before Surgery (g/week)	After Surgery (g/week)
Lean Sham (n=1)	17.00 ± 3.468	4.00 ± 4.980
Lean Denervated (n=1)	14.00 ± 3.601	2.40 ± 5.075
Fa/Fa Sham (n=2)	26.33 ±4.962	1.80 ± 4.994
Fa/Fa Denervated (n=3)	23.83 ± 5.860	-2.80 ± 8.447

The growth rate was assessed weekly by calculating the increase in weight for each animal. Data is shown as the mean and SEM.

4.2. Effect of CSN resection on fasting glycemia

It is very well described that the ZDF rats develop insulin resistance at six-weeks of old, with a rapid progression to a hyperglycemic state. However, the development of hyperglycemia depends from laboratory to laboratory. In our lab, we have screened the animals to submit to denervation at week 10-11th considering fasting glycemia levels over 120 mg/dl. We observed that only 9 animals had glycemias over 120 mg/dl and half of this group was submitted to CSN resection.

In Figure 11 is represented the fasting glycemia levels before and after CSN denervation in lean and Fa/Fa animals. The difference between the Fa/Fa animals and the lean animals grows from 55% on the first week of diet (Lean = 73.8 ± 3.58 mg/dl; Fa/Fa = 123.89 ± 4.19 mg/dl) to 153% in the last week of tests (Lean = 76.7 ± 2.59 mg/dl; Fa/Fa = 194.33 ± 17.07 mg/dl).

Figure 11 shows the effects on glycemia after the surgery. On the 13th week of diet (3 weeks after the surgery), the difference between the Fa/Fa sham group and the Fa/Fa denervated group turns to 8% and the hyperglycemia in the latter seems to be attenuated (Fa/Fa sham = 126.2 ± 3.53 mg/dl; Fa/Fa denervated = 115.00 ± 6.455 mg/dl). 5 weeks after the surgery, the Fa/Fa with CSN resection displayed an astonishing improvement by 22%, comparing with the Fa/Fa animals without CSN resection (Fa/Fa sham = 146.4 ± 21.514 mg/dl; Fa/Fa denervated = 113.75 ± 5.452 mg/dl). The lean animals, though, do not display significant differences following either sham procedures or the CSN resection, showing numbers no more than 6% different.

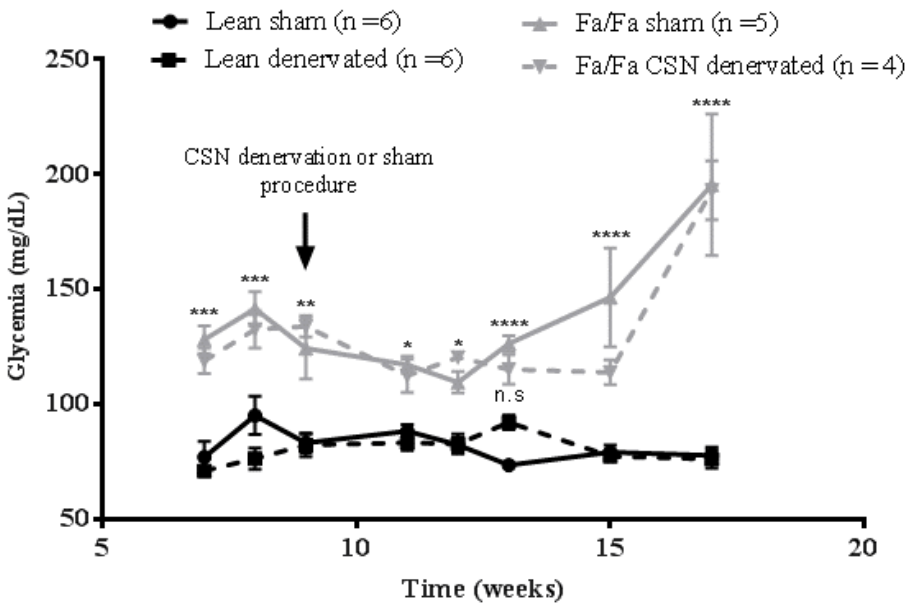

Figure 11 – **Fasting glycemia evolution over time of the early stage animals.**
Surgical procedure for CSN resection is represented by the black arrow. The animals were followed for 7 weeks after the surgery *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 vs lean sham group; n.s. – not significant (Two-way ANOVA Bonferroni test).

The fasting blood glycemia levels in the late stage groups were also measured along the experiment's duration, before each metabolic test. In this figure, it is noticeable the difference between lean animals and Fa/Fa animals, with this difference becoming more evident over time. As it can also be seen in figure 11, it is clear that in ZDF animals' fasting glycemia levels start to increase around the 14-15 week of age. The Fa/Fa animals showed a 120% difference from lean animals on the 15th week of diet, (Lean =74.5 ± 2.5 mg/dl; Fa/Fa = 164.6 ± 19.71 mg/dl) (Figure 12). On the 20th week of diet, this difference increases to 194% (Lean = 92 ± 1.00 mg/dl; Fa/Fa = 271.2 ± 27.601 mg/dl).

Age on lean animals did not modify fasting glycemia as it can be seen in figure 12 (fasting glycemia 20th week of diet Lean sham = 89 mg/dl; Lean denervated = 73 mg/dl).

In the lean animals. the CSN resection showed little effect in the animals' glycemia levels, independently from it being a sham procedure or an actual CSN resection. The Fa/Fa groups, however, started showing a 19% difference after the 2nd week post-surgery (Fa/Fa sham = 276 ± 47 mg/dl; Fa/Fa denervated = 222.33 ± 18.27 mg/dl), which was maintained to the 3rd and final week after surgery (Fa/Fa sham = 308 ± 53

mg/dl; Fa/Fa denervated = 246.67 ± 29.17 mg/dl). Still, the two groups of Fa/Fa animals still presented high fasting glycemia levels.

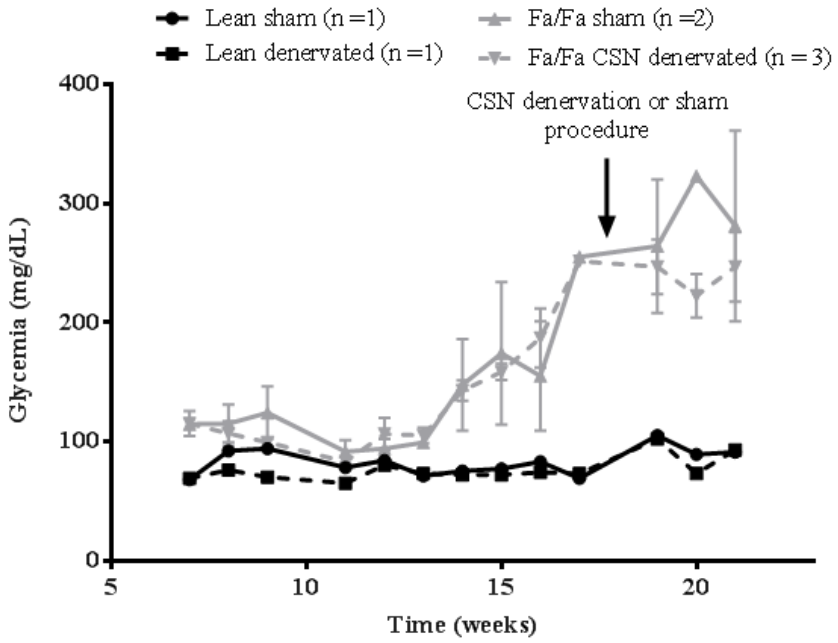

Figure 12 – **Fasting glycemia evolution over time of the late stage animals.**
The black arrow represents the point at which the groups were submitted to the surgeries (either sham procedures or CSN resection). The animals were kept for 3 weeks after the surgery.

4.3. Effect of Fa/Fa phenotype and CSN resection on insulin sensitivity and glucose tolerance

4.3.1. Insulin sensitivity on the Fa/Fa animals is improved by the CSN resection

The animals' insulin sensitivity was evaluated regularly by the ITT, in both sham and denervated groups, lean and Fa/Fa, and expressed as the constant of insulin tolerance test, KITT. As it can be seen in figure 13, lean animals were insulin sensitive (KITT Lean before surgery = 5.01 ± 0.311 % glucose/min). In contrast and as expected, the Fa/Fa phenotype was associated with a clear insulin resistance (KITT Fa/Fa before surgery = 1.13 ± 0.281 % glucose/min). As it can also be seen, age did not modify insulin sensitivity in lean animals for the period of time studied. Also, age did not aggravate the insulin resistance that was observed prior to CSN resection.

CSN resection did not alter insulin sensitivity in the lean animals. In contrast, in the Fa/Fa animals, CSN resection promoted a promising improvement of their insulin

sensitivity with their KITT becoming 158% higher one week after surgery when compared with the values before surgery (KITT Fa/Fa denervated before surgery = 1.56 ± 0.524 % glucose/min; KITT Fa/Fa denervated 1 week after surgery = 4.02 ± 0.248 % glucose/min). This improvement in insulin sensitivity was maintained until the 5th week post-surgery (KITT Fa/Fa denervated 2 week after surgery = 3.59 ± 0.280 % glucose/min; KITT Fa/Fa denervated 3 week after surgery = 3.74 ± 0.316 % glucose/min; KITT Fa/Fa denervated 5 week after surgery = 5.97 ± 0.745 % glucose/min). However, 7 weeks post- CSN resection the reversion of insulin resistance was no longer observable, with the animals becoming insulin resistant again (KITT Fa/Fa denervated 7 weeks after surgery = 1.82 ± 0.699 glucose/min).

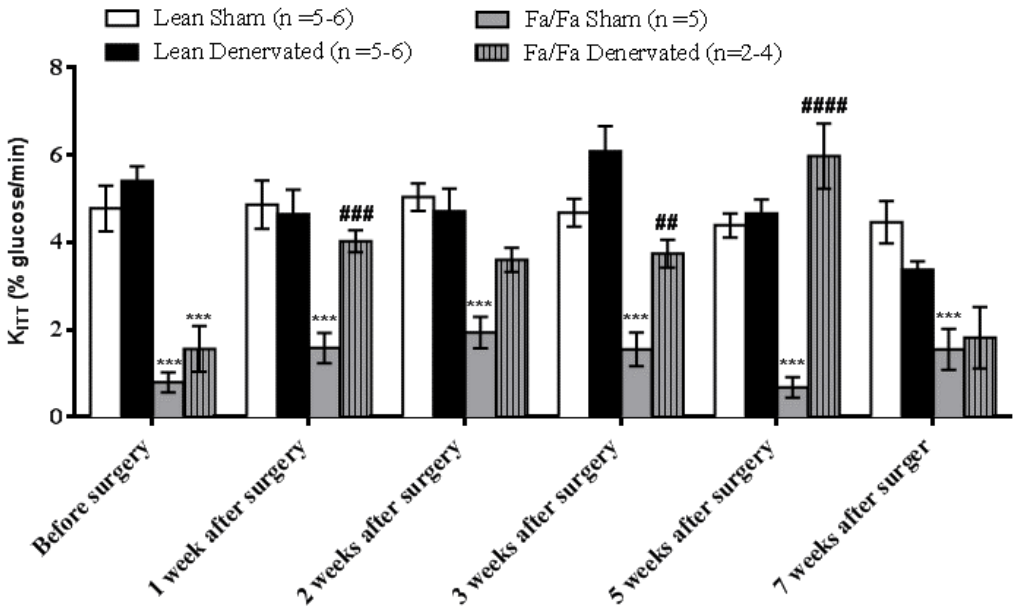

Figure 13 – **Effect of Fa/Fa phenotype and CSN resection on insulin sensitivity in early stage animals.**
Denervation of CSN was performed in the animals at the 10-11th week of age. ***P<0.001 vs lean sham animals; #P<0.05; ##P<0.01; ###P<0.001; ####P<0.0001 vs Fa/Fa sham animals (Two-way ANOVA Bonferroni test).

The late stage animals showed a similarly ameliorating scenario (Figure 14). Before the surgeries, at the 18th week of age, the Fa/Fa animals showed a pronounced insulin resistance, as the KITT values were 82% lower than the lean group (KITT Lean before surgery = 5.25 ± 0.640 % glucose/min; KITT Fa/Fa before surgery = 0.94 ± 0.478 % glucose/min). CSN resection did not modify significantly insulin sensitivity in lean animals. Note also that the groups of lean animals included only one animal per group

and therefore it is difficult to make comparisons. However, CSN resection in Fa/Fa animals increased insulin sensitivity to values similar to the lean values displaying a 141% improvement in insulin sensitivity when compared with values pre-surgery (KITT Fa/Fa denervated before surgery = 1.63 ± 0.294 % glucose/min; KITT Fa/Fa denervated 1 week after surgery = 3.94 ± 0.315 % glucose/min). This increase in insulin sensitivity was maintained throughout the weeks, until the end of the experiment. The Fa/Fa sham maintained insulin resistance (KITT Fa/Fa sham 2 weeks after surgery = 1.53 ± 0.415 % glucose/min; Fa/Fa sham 3 weeks after surgery = 1.59 ± 0.330 % glucose/min; Fa/Fa denervated 2 weeks after surgery = 4.84 ± 1.24 % glucose/min; Fa/Fa denervated 3 weeks after surgery = 3.94 ± 0.195 % glucose/min).

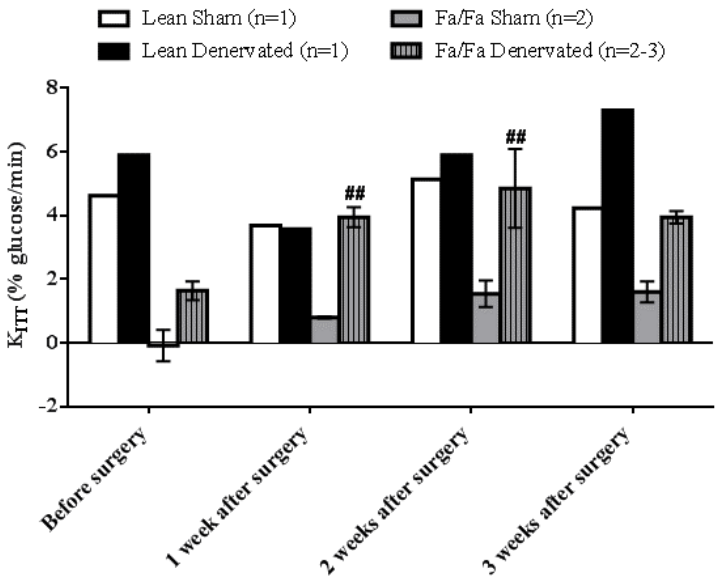

Figure 14 – **Effect of Fa/Fa phenotype and CSN resection on insulin sensitivity in late stage animals.** Animals have been submitted to CSN denervation at the 18th week of age. ##P<0.01 vs Fa/Fa sham animals (Two-way ANOVA Bonferroni test).

4.3.2. Effect of CSN resection on glucose tolerance

Glucose tolerance was evaluated by an oral glucose tolerance test alongside with insulin sensitivity to measure the impact of CSN resection on post-prandial glucose tolerance on the diseased animals, both on the early stage and on the late stage (Figure 15 and Figure 16). When the area under the curve obtained from the glucose excursion curves from the OGTTs is plotted, it is clear that the Fa/Fa animals have an impaired glucose tolerance, when compared with the lean animals, as the AUC increased significantly by 107% (AUC Lean 10th week of age = 18382.25 ± 472.149 mg/dl*min;

AUC Fa/Fa 10th week of age = 38169.22 ± 2666.354 mg/dl*min). CSN resection did not modify glucose tolerance in lean animals in all the time points tested (1, 2, 3, 5 and 7 weeks post-surgery). In Fa/Fa animals, one week post-CSN denervation, no significant statistical differences were observed in glucose tolerance (AUC Fa/Fa sham 1 week after surgery = 34236.20 ± 1421.512 mg/dl*min; AUC Fa/Fa CSN denervated 1 week after surgery = 31052.25 ± 1113.034 mg/dl*min), on the second week after surgery however, the Fa/Fa denervated animals showed a significantly lower AUC than their sham counterparts, by 18% (AUC Fa/Fa sham 2 weeks after surgery = 35962.80 ± 884.480 mg/dl*min; AUC Fa/Fa denervated 2 weeks after surgery = 29254.00 ± 1045.496 mg/dl*min).

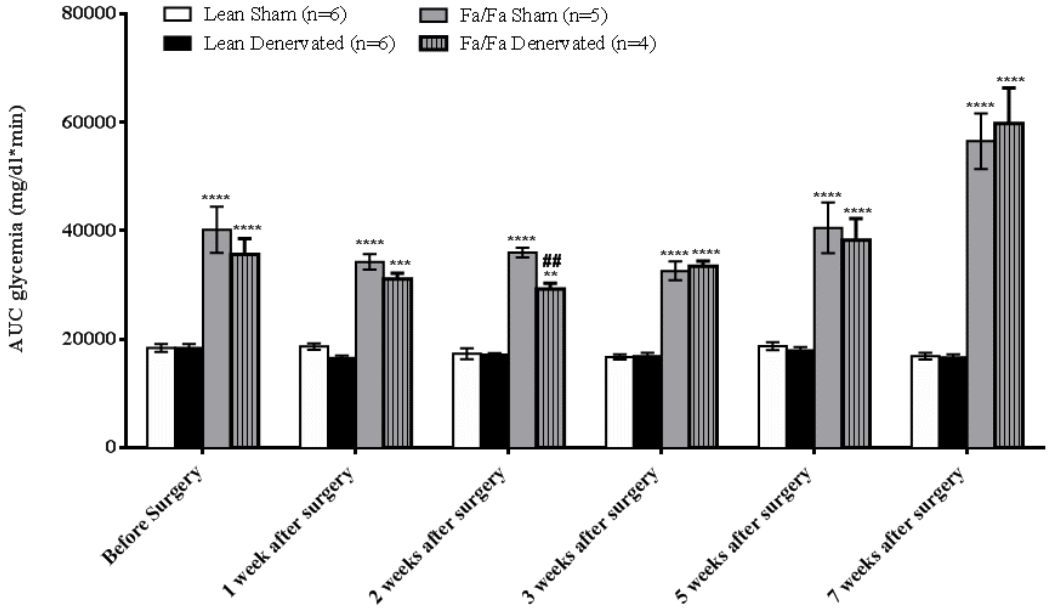

Figure 15 - Effect of Fa/Fa phenotype and CSN resection on glucose tolerance in the early stage animals.
Glucose tolerance is expressed as the area under the curve (AUC) obtained from the glucose excursion curves of the oral glucose tolerance test. **P<0.01; ***P<0.001; ****P<0.0001 vs lean sham animals; ##P<0.01 vs Fa/Fa sham animals (Two-way ANOVA Bonferroni test).

As it happens in the early stage group animals, the Fa/Fa group in the late stage group was glucose intolerant when compared with lean animals, as the AUC obtained from the OGTT were 274% higher (AUC Lean animals 18th week = 17025.50 ± 1582.000 mg/dl*min; AUC Fa/Fa animals 18th week age = 63837.20 ± 5249.851 mg/dl*min) (Figure 16). Also, it can be noted from the comparison of figures 15 and 16, that the Fa/fa animals aggravate glucose intolerance with age, being the values of AUC

at the 18th week of age (Figure 16) higher than at 10th and 17th (Figure 16). CSN resection in lean animals did not modify glucose tolerance (AUC Lean denervated basal values = 18608 mg/dl*min; AUC Lean denervated 1 week after surgery = 18068 mg/dl*min). In Fa/Fa animals, CSN denervation at 18th week of age also did not improve glucose tolerance when comparing with sham animals. The Fa/Fa animals' AUC levels kept climbing from before the surgery until the day of the animals' sacrifice increasing by approximately 5% each week (AUC Fa/Fa denervated basal values = 66532.66 ± 918.789 mg/dl*min; AUC Fa/Fa denervated 1 week after surgery = 64070.00 ± 3507.695 mg/dl*min; AUC Fa/Fa denervated 2 weeks after surgery = 70290.00 ± 2107.184 mg/dl*min; AUC Fa/Fa denervated 3 weeks after surgery = 74288.00 ± 3920.973 mg/dl*min).

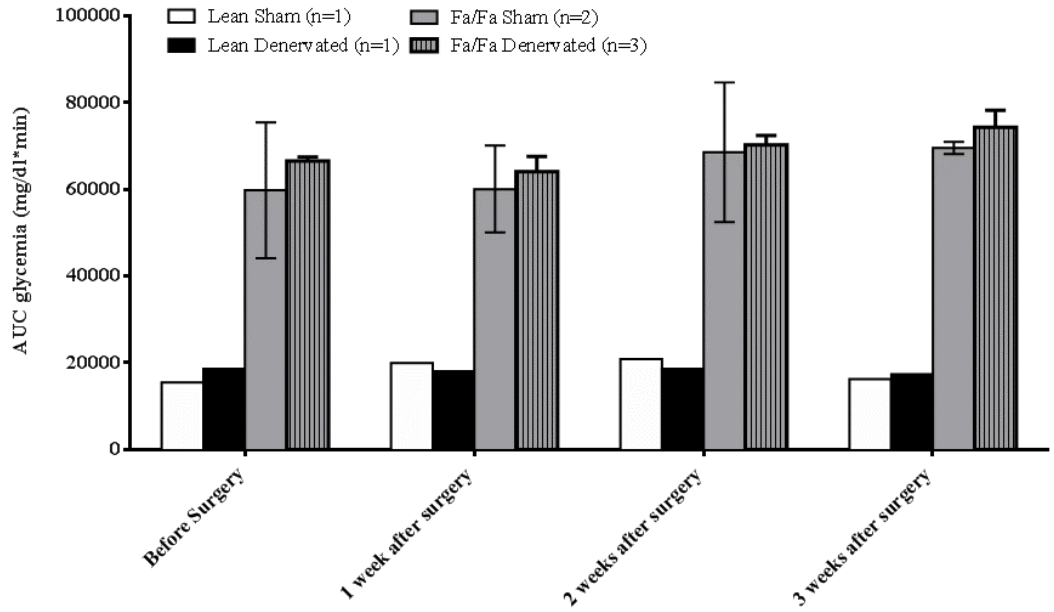

Figure 16 - Effect of Fa/Fa phenotype and CSN resection on glucose tolerance in the late stage animals.
Glucose tolerance is expressed as the area under the curve (AUC) obtained from the glucose excursion curves of the oral glucose tolerance test.

4.4. Effect of Fa/Fa phenotype and CSN resection on basal ventilation and in the responses to hypoxia and hypercapnia

The effect of Fa/Fa phenotype and of CSN resection on basal ventilation and in the responses to hypoxia and hypercapnia, expressed as minute ventilation (V_E), was evaluated through whole-body plethysmography. For the early stage animals'

ventilation was only recorded before the sacrifice procedures (Figure 17a). However, the late stage animals have been evaluated weekly throughout the whole experiment (Figure 17b).

In the early stage animals, CSN resection did not modify V_E both in lean and Fa/fa animals, although there was a slight tendency for the lean sham to have lower basal ventilation values than the remainder groups (V_E Lean sham = 249.2 ± 22.36 ml/min/kg; V_E Lean denervated = 352.0 ± 45.78 ml/min/kg; V_E Fa/Fa sham = 347.6 ± 85.87 ml/min/kg; V_E Fa/Fa denervated = 335.8 ± 96.91 ml/min/kg) (Figure 17a).

Accordingly, in the late stage animals, no significant differences were found when comparing the values of the animals of the different groups before the surgery. The Fa/Fa phenotype did not change the basal ventilation throughout the experiment. CSN resection increased minute ventilation in the lean animals (V_E Lean sham 1 week after surgery = 311.69 ml/min/kg; V_E Lean denervated 1 week after surgery = 375.12 ml/min/kg; V_E Lean sham 2 weeks after surgery = 338.15 ml/min/kg; V_E Lean denervated 2 weeks after surgery = 876.89 ml/min/kg), however these groups were constituted by only one animal and so it is difficult to take conclusions. CSN resection in the Fa/Fa group did not change the basal ventilation (V_E Fa/Fa denervated basal values 234.50 ± 47.464 ml/min/kg; V_E Fa/Fa denervated 1 week after surgery = 277.07 ± 43.276 ml/min/kg; V_E Fa/Fa denervated 2 weeks after surgery = 220.58 ± 10.542 ml/min/kg; V_E Fa/Fa denervated 3 weeks after surgery = 310.92 ± 22.065 ml/min/kg).

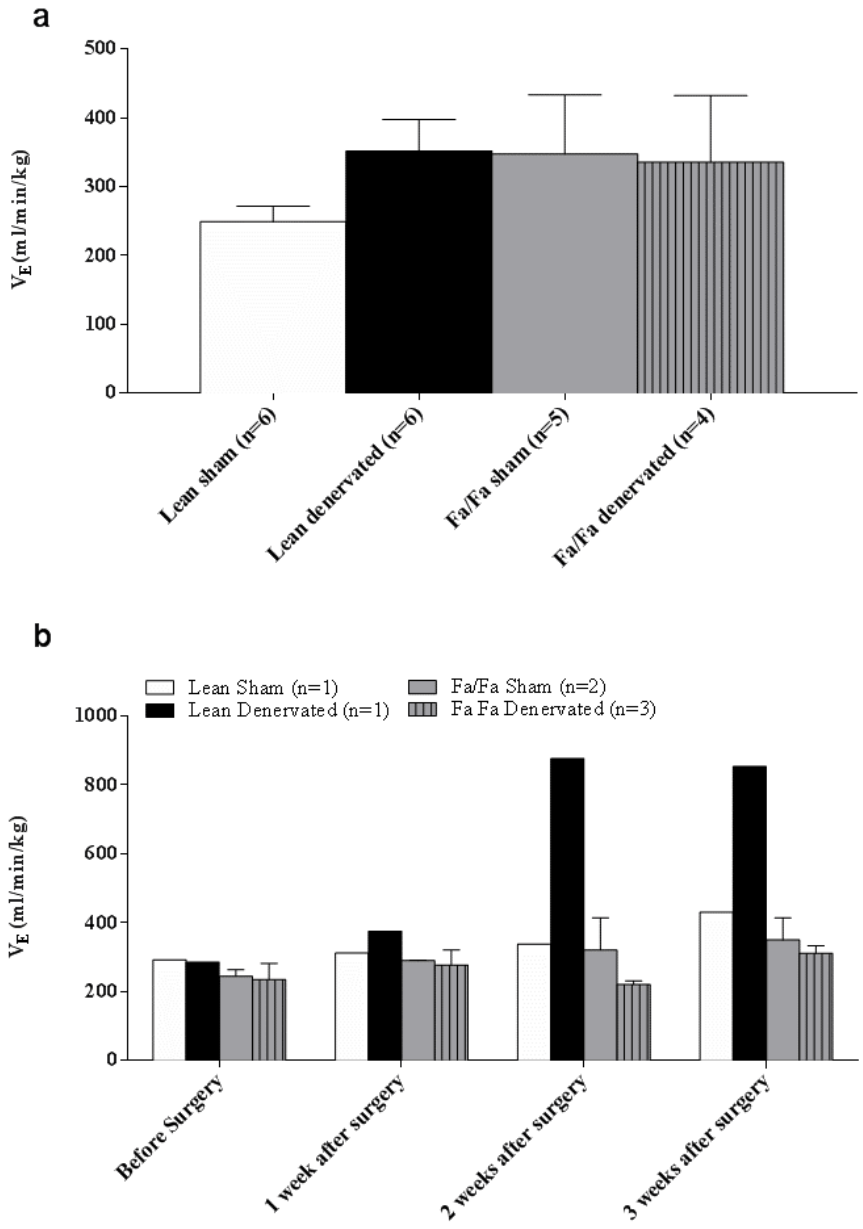

Figure 17 - Effect of Fa/Fa phenotype and CSN resection on basal minute value (V_E).
a) represents the effect of Fa/Fa phenotype and of CSN denervation in V_E in early stage animals recorded before the sacrifice of the animals; b) represents the effect of of Fa/Fa phenotype and of CSN denervation in V_E in the late stage animals, before the CSN resection and 1, 2 and 3 weeks after surgery.

It is very well described that the ZDF animals have decreased hypoxic responses (Schlenker & Farkas, 1995). Additionally, the response to hypoxic ventilatory response is very often used to evaluate the carotid body chemosensititivity (Holton & Wood, 1965). Therefore, to evaluate the effectiveness of CSN resection all groups of animals have been exposed to hypoxic and hypercapnic challenges. As for the basal ventilation

studies, the early stage group was evaluated only before the animals sacrifice (Figure 18) and the late stage group through all the entire experiment (Figure 19).

Figure 18 shows that the Fa/Fa have an impaired response to hypoxia, according to what the literature mentions. The Fa/Fa sham show a hypoxic response 26% lower than the lean sham (Lean sham = 152.2 ± 18.22 %; Fa/Fa sham = 112.6 ± 11.60 %).

The CSN denervation also produced the results expected on the hypoxic response. The lean that were denervated showed a significant decrease of 44% comparing to the lean sham (Lean sham 152.2 ± 18.22 %; Lean denervated = 85.39 ± 8.284 %). Accordingly, the Fa/Fa denervated also had a decrease in the response, being 17% lower than the Fa/Fa sham (Fa/Fa sham = 112.6 ± 11.60 %; Fa/Fa denervated = 92.6 ± 9.75 %) This difference was not significant however. Comparing to the lean sham, however, we see a significant difference of 39% (Lean sham = 152.2 ± 18.22 %; Fa/Fa denervated = 92.6 ± 9.75 %).

Comparing the hypercapnic responses, the Fa/Fa sham had a 30% lower response than the lean sham (Lean sham = 120.4 ± 13.56 %; Fa/Fa sham = 83.6 ± 9.71 %). This difference was not significant.

The animals that were denervated, did not show any significant differences in hypercapnic responses although it seemed there was a tendency for the denervated groups, both lean and Fa/Fa, to display higher hypercapnic responses than their sham counterparts. The lean that were denervated showed a response 14% higher than the sham (Lean sham = 120.4 ± 13.56 %; Lean denervated = 137.2 ± 13.91 %) and the Fa/Fa denervated showed a response 25% higher than the sham (Fa/Fa sham = 83.6 ± 9.71 %; Fa/Fa denervated = 105.1 ± 16.87 %). Again, these differences were not significant.

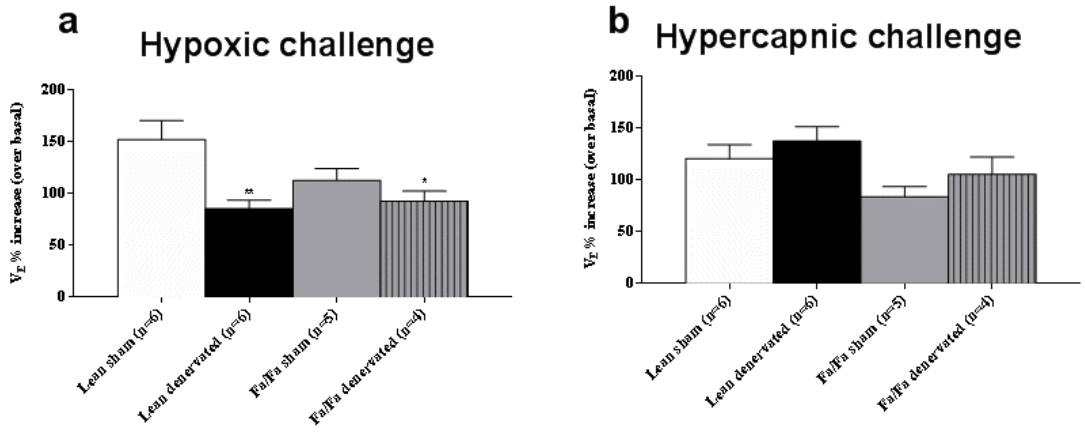

Figure 18 – Effect of Fa/Fa phenotype and CSN resection on hypoxic and hypercapnic ventilatory responses in the early stage animals.
a) Ventilatory responses to hypoxia (10% O_2); b) Ventilatory responses to hypercapnia (5% CO_2). Ventilatory responses were expressed as minute ventilation (V_E) *P<0.05; **P<0.01 vs lean sham animal group (Two-way ANOVA Bonferroni test).

The late stage group results show that, firstly, the hypoxic response of all animals is similar, before any surgery takes place, although there is a tendency for the lean group to have a 22% higher increase of its V_E when challenged with hypoxia than the Fa/Fa group (Lean = 147.5 ± 13.70 %; Fa/Fa = 119.9 ± 7.96 %). On the other hand, on the sham animals, the surgery does not affect hypoxic response, since CB activity is not altered. This is observed both on the lean and Fa/Fa animals. The same was not observed on the denervated animals (Figure 19a).

The percentage of increase in V_E over the normoxic conditions was diminished in hypoxic conditions, especially in the lean group, where it was observed a 403% decrease of response (Lean denervated basal values = 161.2 %; Lean denervated 1 week after surgery = 32.7 %) The Fa/Fa denervated displayed a smaller difference than the lean denervated, with only a 42% difference (Fa/Fa denervated basal values = 116.7 ± 13.09 %; Fa/Fa denervated 1 week after surgery = 81.9 ± 11.36 %).

The percentage of increase in V_E over normoxic conditions in hypercapnic conditions, on the other hand, comparing basal values before the surgery, is 33% lower on the Fa/Fa animals than on the lean (Lean = 112.2 ± 29.89 %; Fa/Fa = 84.23 ± 6.30 %). The lean denervated also shows a 72% lower increase in response to hypercapnia, when comparing to the lean sham, before the surgery (Lean sham = 142.1 %; Lean denervated = 82.3%). This occurrence increases even further after the surgery.

On the first week, all groups are similar but on the second week post-surgery the lean sham distances itself from the other animals, having responses significantly different from the other groups, being 355% higher than the lean denervated and 95 and 164% higher than the Fa/Fa sham and denervated, respectively (Lean sham 2 weeks after surgery = 197.7 %; Lean denervated 2 weeks after surgery = 43.4 %; Fa/Fa sham = 101.2 ± 13.54 %; Fa/Fa denervated 2 weeks after surgery = 74.8 ± 6.69 %). On the third week after surgery, the responses return to being not very distinct from group to group (Figure 19b).

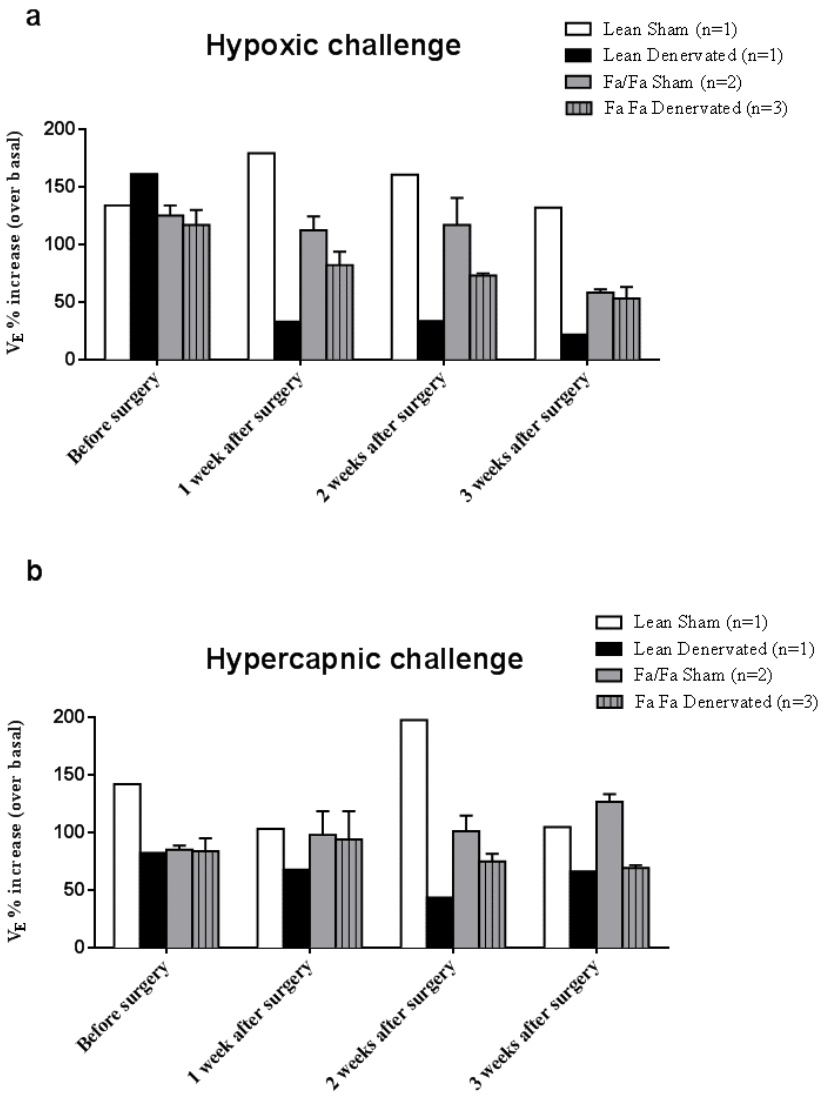

Figure 19 – **Effect of Fa/Fa phenotype and CSN resection on hypoxic and hypercapnic ventilatiory responses in the late stage animals.**
a) Ventilatory Responses to hypoxia (10% O_2); b) Ventilatory Responses to hypercapnia (5% CO_2). Ventilatory responses were expressed as minute ventilation (V_E). Data represents means ± SEM.

4.5. Effect of Fa/Fa phenotype and CSN resection on blood pressure

The systolic (SBP), diastolic (DBP) and mean arterial blood pressure (MBP), heart rate (HR) and p_aO_2 was measured in all groups of animals on the day of their sacrifice (Figure 20). Due to the extreme difficulty of the techniques needed for the catheterization of the femoral artery we have been unable to record blood pressure and heart rate in all the animals.

In figure 20 we can clearly see that Fa/Fa animals exhibit an increased blood pressure than lean animals (MBP lean = 107.45 ± 3.989 mmHg; MBP Fa/Fa = 124.07 ± 6.337 mmHg), however this difference was not statistically different. CSN resection in lean animals significantly increased blood pressure by 20% (MBP Lean sham = 107.45 ± 3.989 mmHg; MBP Lean denervated = 129.50 ± 4.455 mmHg). In contrast, CSN resection in the Fa/Fa animals decreased significantly by 32% SBP, DBP and MBP (MBP: Fa/Fa sham = 124.07 ± 6.337 mmHg; Fa/Fa denervated = 83.90 ± 19.752 mmHg) (Figure 20a). No statistical differences were found in the heart rate of these animals, however CSN resection decreased in a non-statistical manner HR in the Fa/Fa group (HR Fa/Fa sham =317.2 ± 6.79 bpm; HR Fa/Fa denervated = 217.9 ± 58.42 bpm) (Figure 20b).

Fa/Fa animals had the p_aO_2 levels similar to lean animals, however, CSN denervation in Fa/Fa decreased p_aO_2 by 21% (p_aO_2 Fa/FA sham = 86.63 ± 4.162 %; p_aO_2 Fa/Fa denervated = 73.86 ± 6.282 %) (Figure 20c).

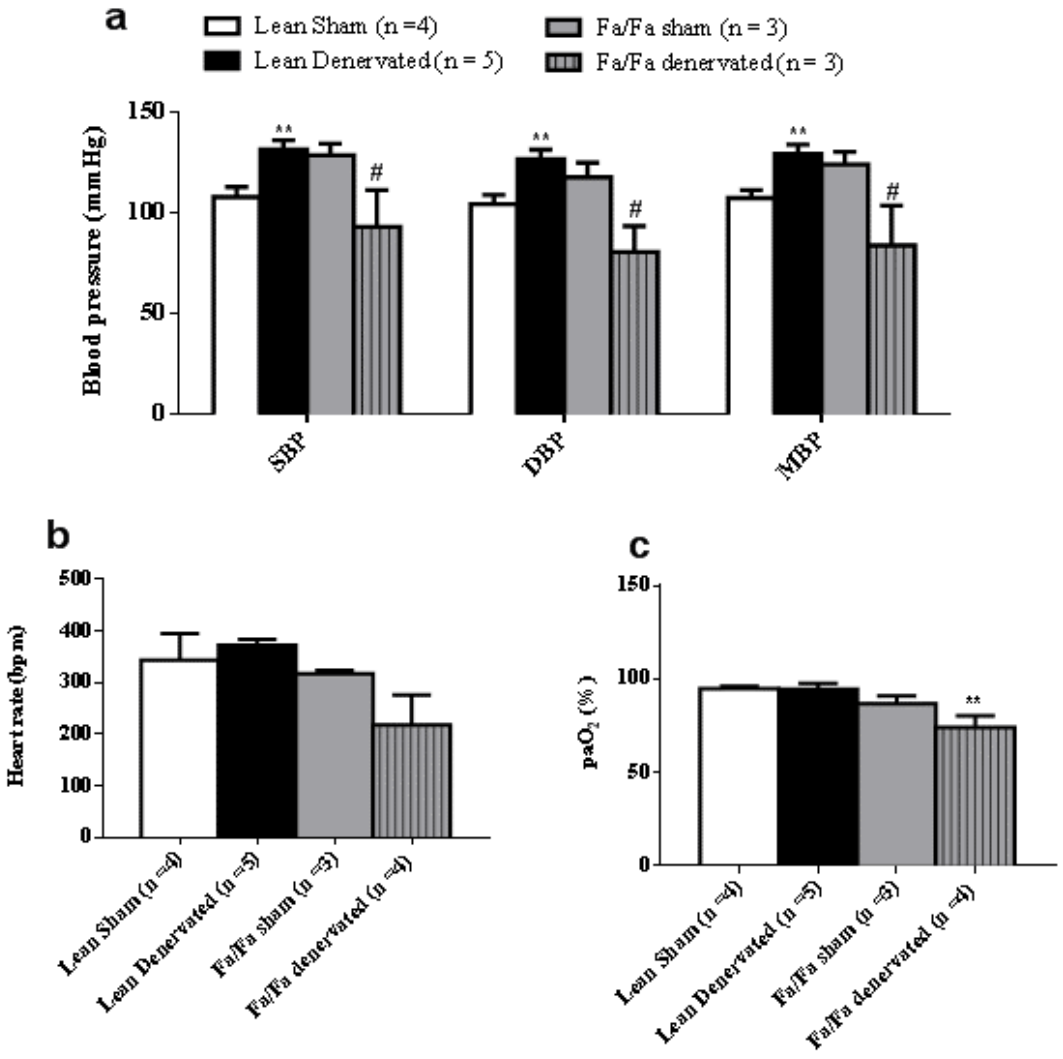

Figure 20 – **Effect of Fa/Fa phenotype and CSN resection on systolic, diastolic and mean blood pressure, heart rate and p_aO_2 in early stage group.**
a) systolic (SBP), diastolic (DBP) and mean blood pressure (MBP); b) Heart rate; c) p_aO_2 of the different groups **P<0.001 vs lean sham animals group; #P<0.05 vs Fa/Fa sham group (One-Way ANOVA test).

The late stage animals also had their blood pressure values recorded upon their sacrifice, although in this group of animals the number of animals was very reduced and we decided to not present the results in the thesis.

4.6. Effect of Fa/Fa phenotype and CSN resection on insulin secretion.

Fasting plasma insulin levels were assessed for the lean and Fa/Fa animals before the surgery, which represents at the 10th week of age for the early stage group and at the 18th week of age for the late stage group and one week and three weeks after the

surgical procedures, sham procedure or CSN resection. At 10th week of age, before the surgical procedure, the early stage Fa/Fa animals exhibit plasma insulin levels 1690% higher than the lean animals with the same age (plasma insulin lean early stage = 51.28 ± 18.348 pmol/l; plasma insulin Fa/Fa early stage = 917.82 ± 52.698 pmol/l) (Figure 21a).

At the 18th week of age, before the surgical procedure, the late stage Fa/Fa animals presented fasting plasma insulin levels 609% higher than the lean animals (plasma insulin lean late stage = 97.10 ± 27.914 pmol/l; plasma insulin Fa/Fa late stage = 688.44 ± 115.784 pmol/l) (Figure 21b). As expected for the ZDF rats, the fasting insulin levels of late stage Fa/Fa animals are already smaller than the early stage Fa/Fa animals (plasma insulin Fa/Fa early stage = 917.82 ± 52.698 pmol/l; plasma insulin Fa/Fa late stage = 688.44 ± 115.784 pmol/l), which suggest than the animals are already progressing to a β-cell failure being this responsible for the appearance of hyperglycemia (Figure 12). This is not so noticeable when we look at figure 21c, when the values of fasting plasma insulin in the Fa/Fa in the early and late stages are separated in sham vs CSN-denervated.

CSN resection in the early stage Fa/Fa animals did not modify significantly fasting insulin levels, however plasma insulin levels seem to show a tendency to increase after CSN resection (plasma insulin Fa/Fa CSN-denervated before surgery = 936.43 ± 108.338 pmol/l; plasma insulin Fa/Fa CSN-denervated 3 weeks after surgery = 1107.29 ± 32.950 pmol/l).

CSN denervation in the late stage Fa/Fa animals decreased fasting plasma insulin levels by 35% the first week and by 40% 3 weeks after CSN denervation, however due to the huge SEM of the Fa/Fa sham the difference between Fa/Fa sham and Fa/Fa denervated was non-significant (Fa /Fa sham late stage 1 week after surgery = 1134.87 ± 4.874 pmol/l; Fa/Fa denervated late stage 1 week after surgery = 738.43 ± 77.158 pmol/l; Fa/Fa sham late stage 3 weeks after surgery= 839.61 ± 370.708 pmol/l; Fa/Fa denervated late stage 3 weeks after surgery = 503.19 ± 10.137 pmol/l).

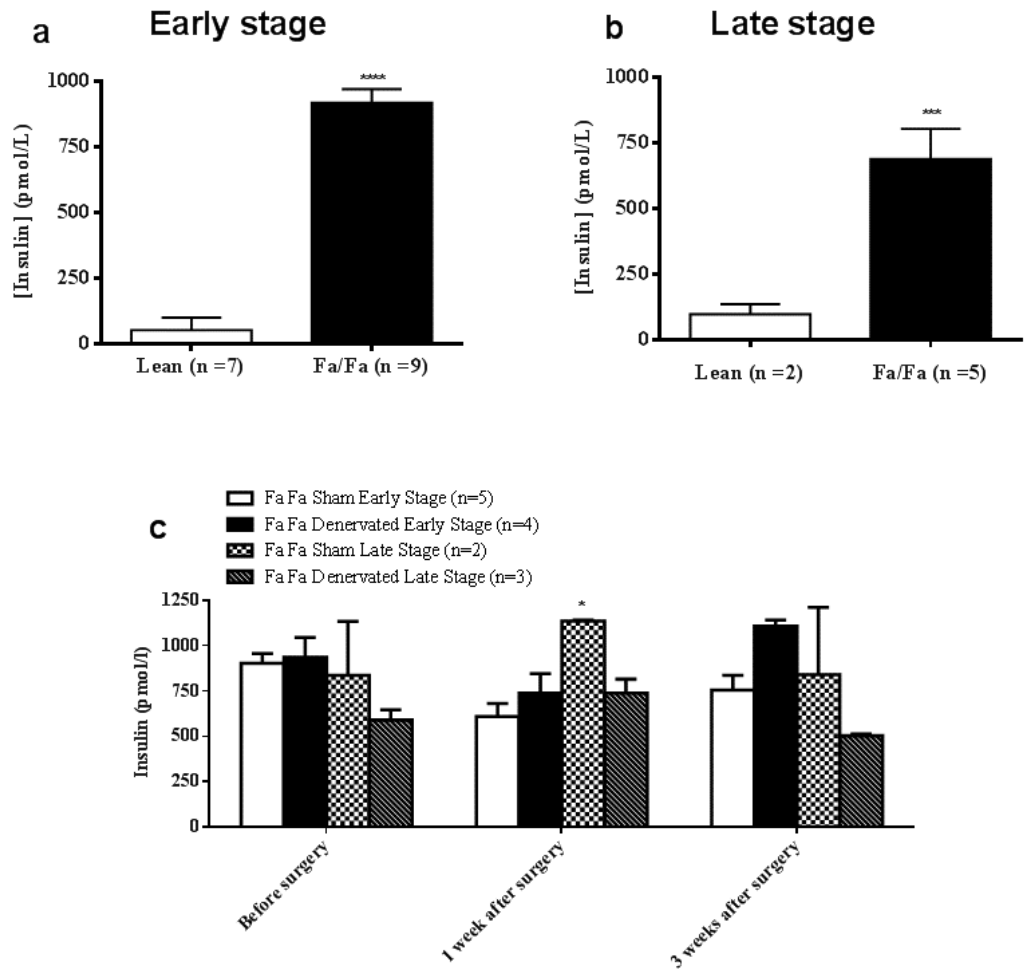

Figure 21 – **Effect of Fa/Fa phenotype and CSN resection on fasting insulin plasma levels**
a) Fasting insulin levels in early stage animals; b) Fasting insulin levels in late stage animals. In white are the lean animals before the surgery and in black the Fa/Fa animals before the surgery. c) effect of CSN resection on insulin levels in early stage Fa/Fa and the late stage Fa/Fa animals. *P<0.05; ***P<0.001; ****P<0.0001 in a) and b) vs lean animal group and in c) vs Fa/Fa sham early stage group (Two-way ANOVA Bonferroni test).

Fasting plasma C-peptide levels have been also assessed to evaluate the endogenous insulin production (Figure 22). Before the surgery, both in the early and in the late stages, Fa/Fa animals show a very significantly higher amount of C-peptide in the plasma. In the early stage, Fa/Fa animals have 686% more C-peptide than the lean animals; the late stage Fa/Fa animals have 183% more C-peptide than the lean (Lean early stage before surgery = 0.55 ± 0.064 nmol/l; Lean late stage before surgery = 0.85 ± 0.003 nmol/l; Fa/Fa early stage before surgery = 4.36 ± 0.187 nmol/l; Fa/Fa late stage before surgery = 2.40 ± 0.194 nmol/l) (Figures 22a and 22b)).

The Fa/Fa animals' C-peptide levels were compared in the weeks following the surgery. Overall, even before the surgery is considered, the late stage Fa/Fa animals show much lower levels than the early stage Fa/Fa animals in the equivalent period. Before the surgery, the difference between the two groups, early and late Fa/Fa animals is 45% (Fa/Fa early stage before surgery = 4.36 ± 0.187 nmol/l; Fa/Fa late stage before surgery = 2.40 nmol/l ± 0.194 nmol/l). The week immediately after the surgery, this difference is still present and, in the third week, the Fa/Fa of the late stage show 50% lower values of C-peptide, when comparing to the Fa/Fa of the late stage (Fa/Fa early stage 3 weeks after surgery = 3.59 ± 0.418 nmol/l; Fa/Fa late stage 3 weeks after surgery = 1.76 ± 0.238 nmol/l).

This difference is most noteworthy when comparing the Fa/Fa sham of the late stage with the other groups. The CSN resection seems to attenuate somewhat the decrease found on the sham animals of the late stage. On the third week after the surgeries. the Fa/Fa denervated animals of the late stage only show a 43% decrease comparing to the Fa/Fa sham of the early stage where the Fa/Fa sham animals of the late stage show a 57% difference compared to the Fa/Fa sham of the early stage (Fa/Fa sham early stage 3 weeks after surgery = 3.32 ± 0.389 nmol/l; Fa/Fa sham late stage 3 weeks after surgery = 1.43 nmol/l; Fa/Fa denervated 3 weeks after surgery = 1.88 ± 0.297 nmol/l).

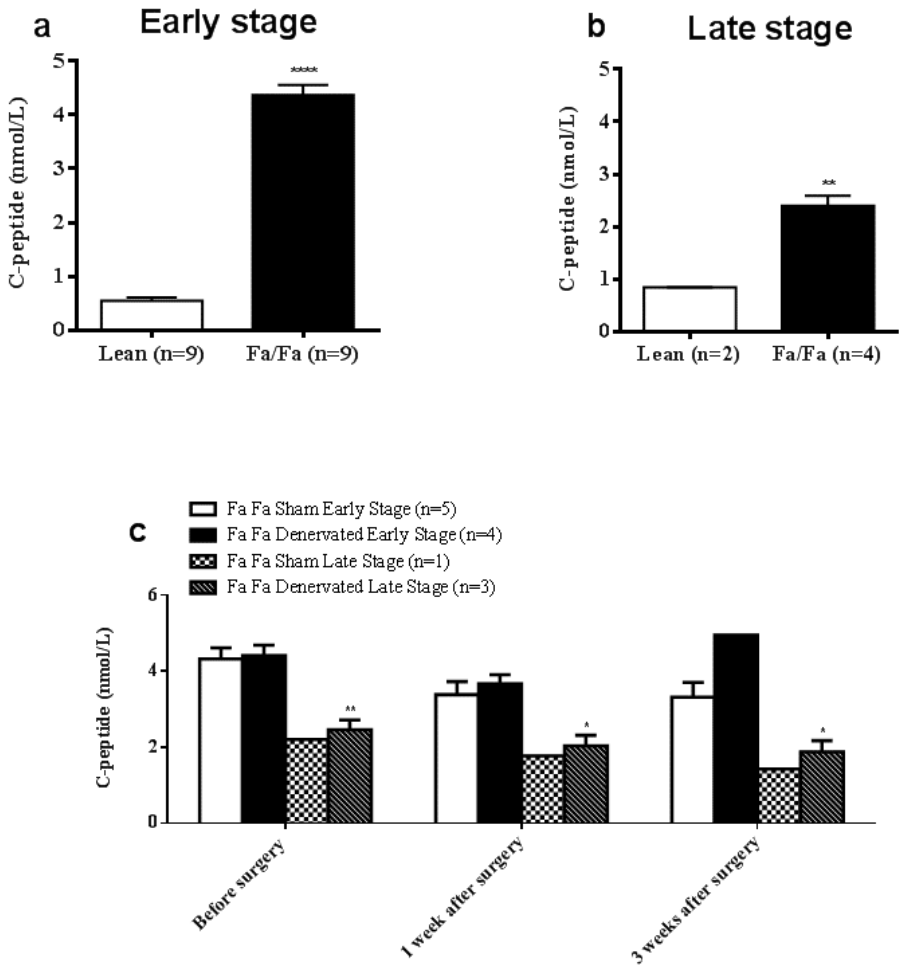

Figure 22 – Effect of Fa/Fa phenotype and CSN resection on fasting C-peptide plasma levels.
a) Fasting C-peptide levels in early stage animals; b) Fasting C-peptide levels in late stage animals. c) effect of CSN resection on fasting C-peptide levels in both early stage Fa/Fa and the late stage Fa/Fa animals. *P<0.05; **P<0.01; ****P<0.0001 in a) and b) vs lean animals and in c) vs Fa/Fa sham early stage group (Two-way ANOVA Bonferroni test)

4.7. Effect of Fa/Fa phenotype and CSN resection on fat mass deposition

Upon the animals' sacrifice, the epididymal, visceral, renal and subcutaneous fat depots were collected and weighed. Total fat amount was then quantified through the sum of the epididymal, visceral, subcutaneous and renal mass and corrected for each animal's weight (Figure 23).

It can be clearly seen, and as expected, since the ZDF rat is a model of obesity, that Fa/Fa animals both in an early stage as well as in a late stage animals exhibit a significant increase in total fat amount by 246% in early stage animals (Lean early stage = 4.52 ± 0.274%; Fa/Fa early stage = 15.64 ± 0.483%) and by 312% in late stage animals (Lean late stage = 3.72 ± 0.13%; Fa/Fa late stage = 15.32 ± 0.543%). CSN

resection did not modify significantly the fat amount in lean early stage animals (Lean sham early stage = 4.28 ± 0.415%; Lean denervated early stage = 4.75 ± 0.366%) as well as in lean late stage animals (Lean sham late stage = 3.85 %; Lean denervated late stage= 3.59%). The same was seen in Fa/Fa animals where CSN resection did not modify the percentage of fat mass in early stage animals (Fa/Fa sham early stage = 15.18 ± 0.757%; Fa/Fa CSN-denervated early stage = 16.23 ± 0.492 %) as well as in late stage animals (Fa/Fa sham late stage = 15.63 ± 1.56%; Fa/Fa denervated late stage = 15.11 ± 0.344%).

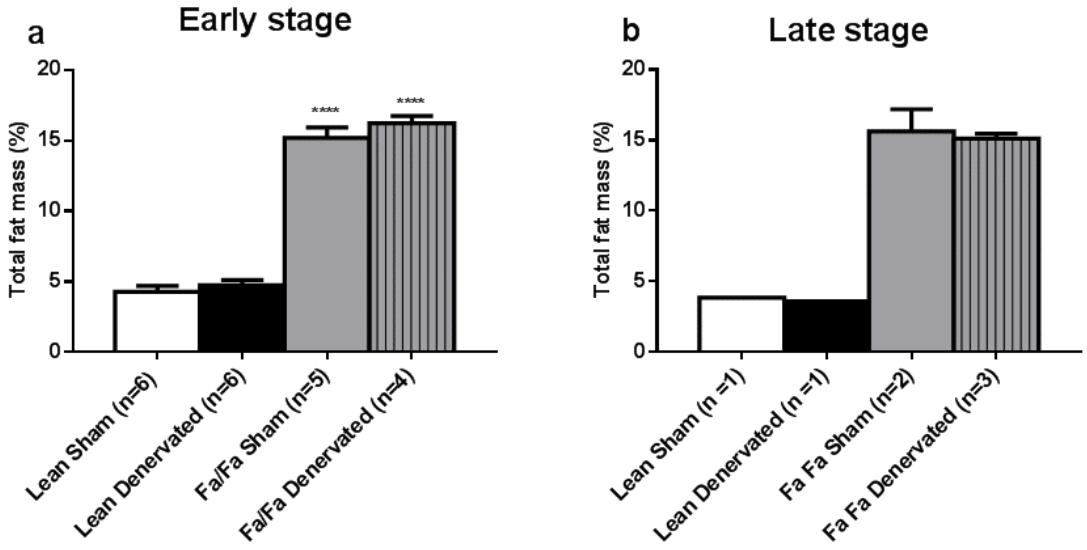

Figure 23 – Effect of Fa/Fa phenotype and CSN resection on total fat mass (%) in early (a) and late stage (b) animals.

Total fat amount was quantified through the sum of the epididymal, visceral, subcutaneous and renal mass and corrected for each animal's weight. ****P<0.0001 vs lean animals (Two-way ANOVA Bonferroni test).

4.8. Effect of Fa/Fa phenotype and CSN resection on liver lipid deposition

Through the use of an optimized version of the protocol described by Elena Olea Fraile (Fraile, 2015), lipid deposition in the liver samples was determined in all groups of animals (Figure 24).

Fa/Fa animals exhibit increased liver lipid deposition by 146% and by 122% in comparison with the lean animals in early and late stage, respectively (Lean sham early stage = 1.48 ± 0.132 %; Lean sham late stage = 1.11 %; Fa/Fa sham early stage = 3.66 ± 0.446 %; Fa/Fa sham late stage = 2.48 ± 0.254 %). The CSN resection did not change

significantly the lipid content both in early and late stage lean animals. However, CSN resection in Fa/Fa decreased by 44% and by 33% liver lipid deposition in early and late stage animals, respectively, to values closer to control values (Fa/Fa sham early stage = 3.67 ± 0.446 %; Fa/Fa denervated early stage = 2.04 ± 0.05 % (Figure 24a); Fa/Fa sham late stage = 2.48 ± 0.254 %; Fa/Fa denervated late stage = 1.67 ± 1.095% (Figure 24b)).

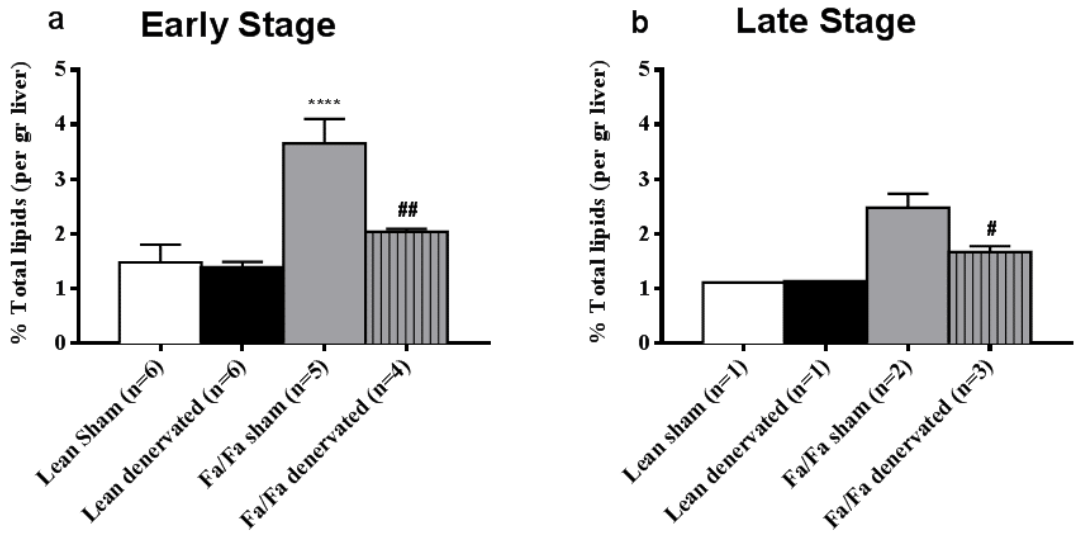

Figure 24 – Effect of Fa/fa phenotype and CSN resection on liver lipid deposition
a) total lipid percentage in the liver in early stage animals; b) total lipid percentage in the liver in late stage animals. Lipid deposition was expressed as Total lipid percentage per gram of liver. ****P<0.0001 vs lean sham animal group; #P<0.05; ##P<0.01 vs Fa/Fa sham group (Two-way ANOVA Bonferroni test).

5. Discussion

In this thesis, we describe for the first time the effects of CSN resection on ZDF (Fa/Fa) rats, a genetic model of obesity and diabetes. In the present work, we found that in early (CSN-denervation performed at 10 weeks of age) and late stage (CSN denervation performed at 18 weeks) animals, that CSN resection did not modify weight gain, glucose tolerance, fat mass deposition and insulin secretion. However, it produced a small decrease in fasting glycemia, totally reversed insulin resistance and decreased lipid deposition in the liver. Additionally, we found that CSN resection on ZDF animals normalized blood pressure in the early stage group.

We have previously demonstrated that the CB is involved in the development of insulin resistance and glucose tolerance and that chronic resection of the CSN prevents and restores insulin sensitivity, glucose tolerance and weight gain in hypercaloric diets models (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017). However, in the literature, there is no record that we could find correlating the CB and this genetic animal model of obesity, the ZDF animal, obtained by the lack of functional leptin receptors. Knowing that 4% of the type 2 diabetes population affected by this pathology have this genetic lack of functional leptin receptors (Clément *et al.*, 1998) it becomes clinically important to investigate the effects of CSN resection on this animal model. Additionally, the ZDF animal is very well described as a good model to study both obesity and diabetes (http://www.criver.com/products-services/basic-research/find-a-model/zucker-diabetic-fatty-(zdf)-rat?loc=PT), especially because in a late stage phase age (after 15 weeks of old), these animals develop a frank hyperglycemia (Figure 12, (Fridlyand *et al.*, 2005; Hempe *et al.*, 2012; Schmidt *et al.*, 2003)), which is not achievable with hypercaloric diets (Conde *et al.*, 2012; Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017). Therefore, to evaluate the impact of a therapeutic on fasting glycemia, the ZDF animal is the best option model.

Effect of CSN denervation on metabolism: weight gain, fasting glycemia, glucose tolerance and insulin sensitivity

Herein we have found, as expected, since the ZDF animal is a model for obesity, that the ZDF animals quickly outgrew their lean counterparts in the first weeks of diet. This kind of data was similar to the one described by Hempe *et al.* (2012), when they

evaluate the ZDF model as an appropriate model to study microvascular late complications. In this study, at eight weeks of age, the ZDF animals outweighed their lean counterparts, weighing, respectively, around 300 and 250 g (Hempe *et al.*, 2012). These results have been also corroborated by the studies of Fridlyand *et al.* (2005) and Pickavance *et al.* (1998) where the animals displayed higher body weights (≈ 300 g in both studies) than those found in the lean animals, around six/eight weeks of age (Fridlyand *et al.*, 2005; Pickavance *et al.*, 1998).

Herein, CSN resection reduced slightly the weight gain in the group of CSN-denervated Fa/Fa animals in the early stage and in the late stage when compared with the Fa/Fa sham animals, although these changes were not significant. The denervation of the lean animals in the early stage also showed some beneficial effects in the first weeks following the surgery. Previously our group has described that CSN denervation decreased significantly weight gain in hypercaloric animal models of insulin resistance and glucose intolerance, the high fat diet rat (3 weeks high fat diet) and the high sucrose diet (4 weeks high sucrose diet) (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017). However, the model used in the present thesis is a genetic model and probably a percentage of the effect cannot be reversible.

The ZDF animals also displayed very high levels of fasting glycemia from 15 weeks old onwards. Since the first study that was performed in this animal model it has been established by many other research groups that this hyperglycemia occurs naturally in all ZDF rats, due to an increase in insulin resistance, and the progressive failure of the pancreas and the inability to cope with the rising need for insulin. For example, Fridlyand *et al.* showed that ten weeks old animals exhibit increased levels of glycemia reaching approximately 216 mg/dl. This is in accordance with our own results, however in our present work we only obtained a frank hyperglycemia reaching those values after 14-15 weeks of old (Fridlyand *et al.*, 2005). Also, Peterson *et al.* (1990) showed their young ZDF animals had very high levels of glycemia, even when comparing with other animal models of obesity, which had shown higher body weights. In this study, Peterson *et al*. described the ZDF animals as having, at ten weeks of age, around 400 mg/dl of glucose, displaying a much more severe hyperglycemia (Peterson *et al.*, 1990). Schmidt *et al.* (2003) likewise described severe hyperglycemia in young ZDF rats, with the lean animals displaying values not much higher than 100 mg/dl whereas the ZDF animals show values as high as 476 mg/dl, almost five times higher than their lean littermates (Schmidt *et al.*, 2003).

The animals submitted to the CSN resection showed very promising improvements, at least in the early stage animals. Three weeks after CSN resection surgery, the Fa/Fa animals started displaying signs of attenuation of hyperglycemia. This is the first time that is described an attenuation of hyperglycemia with CSN resection in ZDF animals. This reversion was attenuated with time, that is, with week progression post-CSN resection. The reason behind this improvement of the glycemia levels may be related to an improved insulin sensitivity of the animals showed in here, thus leading to an increased glucose uptake by the cells, thus lowering the plasma glycemia levels. In fact, the high insulin resistance exhibited by the Fa/Fa animals before surgery and in the sham group was totally reversed one week following CSN-resection an effect that is maintained throughout time until the 5th week post-resection in the early stage animals and at least 3 weeks post-resection in the late stage group. This total reversion of insulin resistance is in agreement with the previous work from our laboratory where we showed in several different hypercaloric models of insulin resistance a complete reversion of insulin sensitivity, like the 3 weeks high fat diet model, the 4 weeks high sucrose diet model or the 14 weeks of high fat-high sucrose diet model (Conde *et al.*, 2016; Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017).

The improvements in both fasting glycemia and in insulin sensitivity in the early stage group disappear with time, and this could be due to a regeneration of the CSN. It is known from previous studies in Wistar rats that CSN starts to regrow 6 days after being cut and usually completely regenerates after 11–12 weeks (Zapata, Stensaas & Eyzaguirre, 1976), however in the present work we see a reversion of CSN-resection effect after 5 to 7 weeks, suggesting that the nerve was already regenerated. In fact, from some observations when dissecting the CBs at the terminal experiment we noticed that 7 weeks post-CSN resection in the early stage group the CSN was already regenerated. This could be due to the high expression of the nerve growth factor that is known to occur in ZDF animals, particularly between 8 and 16 weeks of old (Nisoli *et al.*, 1996).

The reversion of insulin resistance seen herein both in early stage and late stage groups with the CSN resection, could be due to two mechanisms: 1) by a decrease in insulin secretion and therefore a compensatory restoration of the insulin signaling pathways in insulin-sensitive tissues and/or by 2) a direct effect on insulin signaling pathways in insulin-sensitive tissues. Herein we showed that before CSN- resection surgery both groups of Fa/Fa animals – early and late stage groups – were

hyperinsulinemic and also exhibit an increase in C-peptide secretion, suggesting therefore an increase in insulin secretion (figure 21 and 22). These results were in accordance with the works by Pickavance *et al.* who described ZDF animals as displaying very early hyperinsulinemia and high levels of insulin resistance (Pickavance *et al.*, 1998). However, in the present work we haven't seen any consistent effect of CSN resection in normalizing insulin secretion and therefore, we can suggest that the main effect that contributes for the restoration of insulin sensitivity might be a direct amelioration of insulin action on insulin sensitive-tissues, although this needs further exploration.

Additionally, oral glucose tolerance was also very impaired in all the ZDF before the surgery and in the sham animals, both in the early and late stages. This seems to be part of the natural onset of diabetes in these animals. Accordingly, data by Fridlyand *et al.* (Fridlyand *et al.*, 2005) demonstrates that ZDF rats without any kind of treatment show increased values for their AUCs, indicating glucose intolerance (Fridlyand *et al.*, 2005). Furthermore, on semi-fasted ZDF rats, where the animals were offered 50% of their usual 24h food intake one day prior to the test, Paulsen *et al.* (2010) found data consistent with the described in the present thesis and with other authors as well (Clark, Palmer & Shaw, 1983; Paulsen *et al.*, 2010). OGTTS were carried out on the animals at 6, 8, 10, 12, 16, 20 and 26 weeks of age and the results suggested an increasingly more impaired glucose tolerance when the animals were fed the glucose solution. (Paulsen *et al.*, 2010). From the results previously found with the hypercaloric diets (Conde *et al.*, 2016; Sacramento *et al.*, 2017), we were expecting an improvement of glucose tolerance with CSN denervation however, this was not the case with our ZDF animals, whose AUCs kept increasing from week to week. We were expecting, from previous works developed in our laboratory, that the CSN resection induced am increase in GLUT4 and IR expression in insulin-sensitive tissues promoting glucose uptake, and thus increase glucose tolerance (Sacramento *et al.*, 2017). Further studies will be conducted in our laboratory toward understanding the effects of CSN resection on the expression of GLUT4 and IR in adipose tissue and liver samples collected from these animals to evaluate these pathways.

Another possible explanation for the small improvement in weight gain, fasting glycemia and insulin sensitivity is the decrease in caloric intake of these animals, thus promoting a decrease in fat mass and therefore an amelioration of metabolic parameters.

We found here that the caloric and liquid intake of ZDF animals was several times higher than their lean counterparts, from the very early beginning of the study. Similar results were obtained by Pickavance's work, who described that ZDF animals as young as 6 weeks old, display not only higher body weight but also higher caloric intake than the lean animals, this occurring naturally in the animals' lives (Pickavance *et al.*, 1998). The same was seen by Fridlyand *et al.* who described in ZDF rats that they had increased signs of caloric intake compared to the lean controls. The same authors also found that the liquid intake was several times higher than that found for the controls, as a compensation mechanism for the increasing glycosuria, the appearance of glucose in urine (Fridlyand *et al.*, 2005).

CSN resection did not alter caloric and liquid intake in ZDF animals, neither in the early nor late stage animals, when compared to the controls of the same periods. This suggests that the reduction in weight found on the lean denervated animals, in the early stage, and the partial difference developed between the Fa/Fa sham and denervated, must be owed to some other mechanism than the reduction in the caloric intake. Additionally, we found that this reduction in weight cannot be also due to a reduction in fat mass, since in the present study we did not find any effect of the CSN resection on total fat mass amount (figure 22). However, we showed herein that the ZDF animals displayed around 15% of fat (fat/body weight) while the lean animals displayed less than 5%, which is in accordance with the results found by Schummer *et al.* (2008) who showed that in ZDF animals weighing around 390 g a total of 92 g of fat were found contrasting with the 23 g of fat found in lean animals with 356 g (Schummer *et al.*, 2008). This corresponds to about 23% of fat in the ZDF animals and 6% in the lean, which is relatable to the results found by us.

Effect of Fa/fa phenotype and of CSN resection on basal ventilation and ventilatory responses to hypoxia and hypercapnia

Whole-body plethysmography was performed on the animals and showed that both lean and ZDF rats do not reveal any significant differences between them in terms of V_E values, both in the early and late stage animals, although there is a slight tendency towards the Fa/Fa sham in the early stage to display higher ventilation values (figure 17). However, there are no results, at the present, regarding whole-body plethysmographies and ZDF rats. There are though, some results comparing two

different strains, one of them closely related to the ZDF: the Zucker obese rat (with high breathing frequencies and a leptin-related deficiency), and the Brown Norway rat (a non-obese strain with low breathing frequency and normal alleles for the leptin receptor). Their respective R_f, V_T and V_E were analyzed by Iyengar and coworkers (2004). In this study, Zucker obese display significantly higher values of ventilation than the Brown Norway (Iyengar *et al.*, 2004).

There were, inclusively, results regarding the hypoxic (10% O_2) and hypercapnic (7% CO_2) responses of these two different strains and it was found that the Zucker animals displayed significantly higher values for R_f and V_E than the Brown Norway rats, in response to hypoxia. Also, the Zucker animals displayed significantly higher responses reflected by increases of the R_f and V_E than the Brown Norway rats to hypercapnia (Iyengar *et al.*, 2004). However, it is consensual in the literature that ZDF animals exhibit decreased responses to hypoxia and hypercapnia in comparison with lean animals, being this due to the lack of the functional leptin receptors, since leptin administration reverses the lack of hypoxia and hypercapnia responses commonly encountered in animal models with absence of the functional hormone (O'Donnell *et al.*, 1999; Tankersley *et al.*, 1998).

As we can see in the present work the reduction in the ventilatory responses to hypoxia and hypercapnia was perfectly seen in the ZDF animals (figure 18 early stage animals and 19 late stage), although it was not statistically significant. Probably, if we increase the number of animals per group we will obtain a statistically significant difference. These results are completely in agreement with the results described in the literature (O'Donnell *et al.*, 1999; Tankersley *et al.*, 1998).

The CSN resection, in both stages, did not significantly alter basal ventilatory parameters. However, the unexpected increase in V_E found for the lean denervated animal of the late stage was noted, however we cannot take any conclusions since this group only included one animal. Since there are no studies following CSN denervated ZDF and lean animals in the literature, further studies are necessary to confirm these results on ventilation.

On the other hand, CSN resection, as expected, decreased significantly the ventilatory responses to hypoxia, as hypoxia is the classical stimulus for CB activation (Gonzalez *et al.*, 1994). This reduction was conserved as far as the third week after the surgery, on the late stage animals, both lean and Fa/Fa. Usually, there is a compensatory adaptation by the central nervous system to compensate the lack of CB-hypoxic

mediated responses, however 3 weeks in this strain of animals does not seem to be enough time for the central nervous system to compensate the lack of hypoxic responses. Hypercapnic responses were not modified by CSN resection, a result that was expected since the CB plays a small role in mediating the hypercapnic ventilator responses (Gonzalez *et al.*, 1994).

Effect of Fa/Fa phenotype and CSN resection on cardiovascular parameters

Apart from being an obesity and diabetes model, the ZDF animals are known to develop also hypertension (http://www.criver.com/products-services/basic-research/find-a-model/zucker-diabetic-fatty-(zdf)-rat?loc=PT). Herein, we confirmed those results as the BP values found in this work for the early stage Zucker animals showed that the Fa/Fa sham animals have a slightly higher BP than the lean controls (figure 20). These results are also in agreement with the study by Allwood *et al* (2015), where they evaluated 14-16 weeks old anesthetized lean and ZDF animals' blood pressures and showed values for the SBP of approximately 112 and 133 mmHg, respectively, being the difference significantly different with a $P<0.05$. These results confirm that ZDF animals are hypertensive such as what was found in the present thesis (Allwood *et al.*, 2015).

CSN resection allowed the reduction of the naturally occurring hypertension of the ZDF animals, in the early stage animals. The Fa/Fa submitted to CSN denervation had their MBP values decreased to values similar to the lean animals. This was expected as a role for the CB in the pathogenesis of essential hypertension was observed in spontaneously hypertensive rats since these animals were submitted to bilateral CSN denervation exhibited a delay in the development and maintenance of hypertension, a reduction in sympathetic vasomotor tone and a decreased renal sympathetic activity (Abdala *et al.*, 2012; McBryde *et al.*, 2013). Additionally, in our laboratory we also showed that CSN resection prevents the development of hypertension and normalizes BP in hypercaloric animal models (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017).

We also found that heart rate also decreased beyond control values. Furthermore, p_aO_2 became significantly lower in the Fa/Fa denervated animals. This could be explained through the reduced activity of the CB itself since its primary function is to increase ventilation in response to hypoxia. If this activity is impaired, then perhaps the arterial oxygenation could also be reduced.

Effect of Fa/Fa phenotype and CSN resection on liver lipid deposition

Our studies involving the determination of the total lipid content of the liver using the method developed by Elena Olea Fraile (Fraile, 2015) revealed that the ZDF animals have a much fattier liver, than the lean controls. Forcheron *et al*. (2009) in their studies confirmed these findings when analyzing liver samples from ZDF animals and lean controls at 7, 14 and 21 weeks of age, with a protocol sharing the use of chloroform and methanol (1:2), for the separation of the organic phase, and later determining the content of TAG content enzymatically. What they found was that at 7 weeks, the ZDF rats had 6 times more TAG molecules in the liver than the controls; at 14 and 21 weeks, the situation was similar (Forcheron *et al.*, 2009). Sreenan *et al*. (1999) describes results consistent with both ours and Forcheron *et al.*'s. In their study, they isolated ZDF and lean rats' liver islets and separated the fatty content from the rest using also chloroform and methanol and determining total TAG content enzymatically. The total TAG content was, again, much higher on the ZDF animals than on the lean, accordingly to what was found in our work (Sreenan *et al.*, 1999).

We describe here, unprecedently, the effects of the CSN resection on liver lipid content of ZDF animals and their lean counterparts. What was found was that the fat content was significantly lower on the denervated animals, though it did not change the lipid content of the lean animals' livers. It has already been described that the CSN resection can improve the lipid profile of diet-induced obesity models in Sacramento *et al.* (2017) and Ribeiro *et al.*'s (2013) studies although the mechanism remains to be clarified (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017). This could bring a positive change to a known comorbidity of diabetes and obesity: nonalcoholic fatty liver disease. This condition is known as the excessive accumulation of fat in the liver in the absence of alcohol consumption (Forcheron *et al.*, 2009). The fact that we describe here that CSN resection decreases lipid accumulation in liver and that it improves the lipid profile of diet-induced obesity models (Sacramento *et al.*, 2017) maybe it could be used as an effective therapeutic strategy to treat this comorbidity.

Limitations of the study

Many results were produced here for the first time, especially regarding the effects of the CSN resection on the ZDF phenotype. However, some details can be improved,

namely, regarding the late stage's control groups' low numbers since in the lean animals of the late stage, both sham and denervated, the groups showed one animal each. When comparing the ZDF animals of the sham and the CSN resection groups it was hard to make any reliable conclusions. Therefore, the population of the study should be increased.

Also, to study the insulin-sensitive tissues' insulin signaling pathways and the expression of UCP1 by BAT, some Western blots were made during this thesis. However, since the quality of these was not optimal and no conclusive results were found within the time allotted for the conclusion of the thesis, they have not been presented herein. Further work will be done in this line in order to better understand the effects of CSN resection on the ZDF phenotype.

6. Conclusions

Obesity has been established as a very serious public health issue and the number of obese and overweight people has doubled in the last several decades. The occurrence of diabetes and other comorbidities including cardiovascular problems and even cancer urge the need for the development of efficient therapies that can control the emerging number of obese people in the world.

In this study, CSN resection was performed for the first time on ZDF rats with obesity and established diabetes, to evaluate the impact of modulation of CB activity for the treatment of these pathological conditions.

We showed for the first time, the powerful effects of the modulation of CB activity to treat insulin resistance, decrease fasting glycemia and reduce lipid deposition in the liver, in our genetic model of obesity. However, it was not as effective in treating obesity *per se* as the animals had no significant changes in weight, glucose tolerance, fat mass deposition and insulin secretion.

We suggest that the modulation of CB activity in obesity and type 2 diabetes, due to the lack of leptin receptors, might not be sufficient to treat these conditions.

7. Future Work

The work that is most needed to complete the present thesis is the increase in the number of animals in the late stage group, where the lean groups only had one animal each.

Apart from this there are still several questions that need to be clarified in the line of this project. For this reason, in the future, we will explore:

- The role of inflammation on the adipose tissue in obesity, including also the role of hypoxia in this tissue;
- Insulin signaling pathways in insulin-sensitive tissues, such as the liver, muscle and adipose tissue, through Western blot, by investigating GLUT4 and IR expression, among other markers;
- Zucker animals' lipid profile, and the effect that CSN resection has on total blood lipid profile;
- Thermogenic activity of BAT, through enzymatic assays;
- Evaluate the expression of browning markers like UCP1 and PGC-1α, on BAT and WAT.

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