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Leptin and Sertoli cells mitochondrial bioenergetics

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Resumo

As doenças metabólicas, entre elas a obesidade, são um dos grandes desafios do século XXI. A incidência da obesidade em indivíduos do sexo masculino em idade reprodutiva tem vindo a aumentar, e as previsões indicam que esta tendência se irá manter. Na direção oposta está o decréscimo dos parâmetros de fertilidade destes indivíduos, o que se tem vindo a refletir no aumento da recorrência de casais a clínicas de fertilidade. O fator masculino de forma isolada ou em conjunto com o fator feminino está presente em um terço dos casais que procuram tratamentos de fertilidade. Desta forma, não é surpreendente a queda abrupta dos parâmetros espermáticos que se tem vindo a verificar nas últimas décadas, atingindo valores preocupantes.

Os indivíduos com excesso de peso e obesos apresentam uma disfunção hormonal, relacionada principalmente com a presença de valores elevados de leptina. Além das funções já descritas a nível hipotalâmico, esta hormona apresenta diversas funções em tecidos periféricos. No entanto, apesar de já ter sido descoberta há duas décadas, os seus efeitos no trato reprodutor masculino, principalmente nas células de Sertoli, continuam por desvendar. Recentemente, diversos estudos demonstraram a capacidade da leptina de modular as dinâmicas mitocondriais, incluindo a sua biogénese e funcionamento em vários sistemas celulares, incluindo células cancerígenas. Neste trabalho, estudamos o efeito da leptina na proliferação e atividade metabólica das células de Sertoli de rato. Também avaliamos os efeitos da leptina na fisiologia mitocondrial, particularmente nos níveis dos complexos mitocondriais, níveis de ARN mensageiro (mRNA) de genes envolvidos na biogénese mitocondrial e o potencial mitocondrial de membrana. Para efeitos comparativos, e tendo em conta resultados prévios do nosso grupo, também avaliamos os efeitos da leptina nos níveis de mRNA de genes envolvidos na biogénese mitocondrial e nos níveis dos complexos mitocondriais em células de Sertoli humanas.

Os nossos resultados indicam que a leptina modula a atividade metabólica e função mitocondrial nas células de Sertoli de rato na concentração de 50 ng/mL, uma concentração que está presente em indivíduos com obesidade mórbida. Estes resultados sugerem que altas concentrações de leptina, como esta que é encontrada nestes indivíduos, modula a função mitocondrial nas células de Sertoli de rato o que pode representar um mecanismo novo através do qual a leptina contribui para a subfertilidade e infertilidade induzida pela obesidade em indivíduos do sexo masculino. No entanto, a exposição à leptina não teve efeito em vários parâmetros da fisiologia mitocondrial. Os níveis de mRNA de genes envolvidos na biogénese mitocondrial e os níveis dos complexos mitocondriais não apresentaram alterações, o que fortalece a hipótese de que a leptina modula a função mitocondrial e não a sua

fisiologia. Nas células de Sertoli humanas, os níveis de mRNA da Sirtuina 1 (*SIRT1*) apresentaram alterações no grupo exposto a uma concentração de 50 ng/mL de leptina. Os níveis proteicos do complexo mitocondrial II também apresentaram alterações no grupo exposto às concentrações de 5 e 50 ng/mL, ao passo que nas células de Sertoli de rato isto não se verificou, o que indica que existem respostas à leptina nas células de Sertoli que são dependentes da espécie. Estas diferenças, principalmente nos níveis de mRNA da *SIRT1*, podem representar um mecanismo novo através do qual a leptina afeta o controlo metabólico da espermatogénese, com possíveis consequências nas células de Sertoli humanas.

Palavras-chave

Leptina; Obesidade; Células de Sertoli; Espermatogénese; Infertilidade; Mitocôndria.

Resumo alargado

O sistema reprodutor masculino é constituído por diversas estruturas que podem ser divididas em órgãos sexuais primários e órgãos sexuais secundários. Os testículos fazem parte da primeira categoria, sendo considerados os elementos centrais do sistema reprodutor masculino. Eles são responsáveis pela síntese de esteroides, os quais estão envolvidos no desenvolvimento dos órgãos sexuais secundários, e pela produção dos gâmetas masculinos, os espermatozoides. Nos testículos estão presentes diversos tipos celulares com funções específicas. As células de Sertoli desempenham um papel fundamental no desenvolvimento funcional dos testículos e no processo de produção de gâmetas masculinos, a espermatogénese. Estas células fornecem o suporte físico e nutricional às células germinativas nas diversas fases da espermatogénese, desde espermatogónias até atingirem o estado de espermatozoides, os quais são posteriormente libertados no lúmen dos túbulos seminíferos. Nesta fase, os espermatozoides são células diferenciadas, mas incapazes de fertilizar o óvulo. Essa capacidade é adquirida nas seguintes etapas de maturação e capacitação que ocorrem no epidídimo e ao longo do trato reprodutor feminino, respetivamente. Além do suporte físico e nutricional, as células de Sertoli também produzem lactato em elevadas quantidades, que funciona como fonte de energia para as células germinativas. Neste processo, as células de Sertoli metabolizam a glicose em piruvato e este depois é metabolizado em lactato, num processo conhecido como glicólise. Esta cooperação metabólica entre estas as células de Sertoli e as células germinativas é fundamental. No entanto, o ambiente dos túbulos seminíferos é suscetível a variações de hormonas e metabolitos, o que torna a regulação desta cooperação metabólica crucial para uma espermatogénese bem-sucedida. A obesidade, caracterizada por uma forte desregulação a nível hormonal especialmente ao nível do eixo leptina-grelina, é uma patologia cuja proporção pandémica tem vindo a preocupar especialistas e políticos. A obesidade em indivíduos do sexo masculino em idade reprodutiva tem também acompanhado esta tendência exponencial de crescimento. Ao mesmo tempo, a qualidade da reprodução desses indivíduos tem seguido uma tendência inversa. De facto, vários estudos têm relacionado o aumento do índice de massa corporal com o decréscimo dos parâmetros reprodutivos como a concentração espermática e a motilidade. No entanto, apesar das relações estabelecidas entre a obesidade e a diminuição do potencial reprodutivo masculino, pouco se sabe sobre os mecanismos responsáveis por essas alterações.

A leptina, produzida principalmente nos adipócitos, é uma hormona com uma estrutura semelhante a algumas citocinas estando envolvida na sensação de saciedade. Em indivíduos com um índice de massa corporal normal, valores de leptina elevados desencadeiam uma reação que promove a redução do consumo de energia na forma de

comida e promove o gasto energético. Em indivíduos obesos este mecanismo está desregulado o que provoca a acumulação de leptina sem ocorrer o desencadeamento de uma resposta proporcional. Isto acontece devido a um fenómeno designado por resistência à leptina. Além da sua função como regulador de apetite, vários estudos já conseguiram identificar diversas ações da leptina em tecidos periféricos. No entanto, apesar de já ter sido descoberta há duas décadas, ainda pouco se sabe sobre os seus efeitos no trato reprodutor masculino, particularmente nas células de Sertoli. Nos últimos anos, diversos estudos têm-se focado no papel da leptina na mitocôndria, particularmente em células cancerígenas. O metabolismo das células cancerígenas tem diversos pontos de contacto com o metabolismo das células de Sertoli, uma vez que ambas priorizam a glicólise em detrimento da fosforilação oxidativa. Alguns desses estudos demonstraram que a leptina tem a capacidade de modular a função mitocondrial, incluindo a sua biogénese.

O objetivo deste trabalho foi o de investigar os efeitos da leptina na proliferação e atividade metabólica de células de Sertoli de rato. Além disso, também foram avaliados os efeitos da leptina na fisiologia mitocondrial, tanto nos níveis de ARN mensageiro (mRNA) de marcadores da biogénese mitocondrial como no potencial de membrana e nos níveis de proteína dos complexos mitocondriais. Para isso, foi usada uma linha de células de Sertoli de rato (SerW3), cultivadas na ausência e na presença de concentrações crescentes de leptina de forma a mimetizar diferentes condições fisiológicas. Foram usadas três concentrações de leptina: 5 ng/mL, um valor encontrado em ratos e humanos com um índice de massa corporal considerado normal; 25 ng/mL, um valor reportado na literatura como estando presente em modelos de obesidade animal e em humanos obesos; e 50 ng/mL, um valor encontrado em humanos com obesidade mórbida. Apesar de não existirem dados sobre qual a concentração de leptina presente em ratos com obesidade mórbida uma vez que este modelo animal não existe, achamos pertinente avaliar os efeitos desta concentração em ratos. Para efeitos comparativos, e tendo em conta os resultados obtidos recentemente pelo nosso grupo em células de Sertoli humanas expostas a leptina, também foram avaliados os efeitos da leptina nos níveis de mRNA de marcadores da biogénese mitocondrial e nos níveis de proteína dos complexos mitocondriais em células de Sertoli humanas. Para tal foi usada uma linha de células de Sertoli humanas cultivadas nos mesmos parâmetros que as células de Sertoli de rato.

Os resultados obtidos revelam que a leptina modula a função mitocondrial numa concentração de 50 ng/mL em células de Sertoli de rato, mas não tem efeitos nos restantes parâmetros avaliados da fisiologia mitocondrial. Isto sugere que elevadas concentrações de leptina, como acontece em indivíduos com obesidade mórbida, induz disfunção mitocondrial. Os nossos resultados demonstram que não há alterações a nível da biogénese e expressão dos complexos mitocondriais nas células expostas a essa concentração de leptina, pelo que sugerimos alterações na funcionalidade, o que terá possíveis implicações na

espermatogénese. Os resultados obtidos em células de Sertoli humanas revelam que estas e as células de Sertoli de rato respondem de forma diferente à exposição a leptina. Esta resposta dependente da espécie, já reportada quando analisados outros parâmetros de funcionamento biológico, evidencia as cautelas que se devem ter quando se discutem trabalhos em animais e se tenta fazer a translação para humanos. Nas células de Sertoli humanas foram obtidas diferenças nos níveis de mRNA da Sirtuina 1 (*SIRT1*) o que, devido ao seu papel no controlo metabólico e na glicólise, suporta trabalhos anteriores do grupo em que foi demonstrado que a leptina afeta a glicólise nas células de Sertoli humanas e consequentemente a espermatogénese. No entanto, mais estudos são necessários para estudar esta via de sinalização e a sua relevância nestes indivíduos em que a concentração de leptina está muito elevada. Assim, a leptina atua sobre as células de Sertoli, apresentando modos de ação distintos entre espécies de rato e humanos, sendo que a disfunção nos níveis normais desta hormona pode comprometer o suporte nutricional da espermatogénese.

Abstract

Metabolic diseases, such as obesity, stand as one of the greatest challenges of the 21st century. Obesity in reproductive-age men has risen and is expected to continue to increase. In an inverse direction, fertility is decreasing in those men and it largely contributes for the high demand of fertility treatment by couples in modern societies. The male factor alone or in combination with female factor is present in 1/3 of the couples seeking for fertility treatment. In fact, sperm parameters are on a downward spiral during the last decades reaching worrying levels.

In overweight and obese individuals, there is a hormonal dysfunction, particularly in leptin levels that are heavily increased. Besides the well-described functions at the hypothalamic level, leptin acts in several peripheral tissues. Although leptin has been on spotlight since its discovery, its effects in the male reproductive tract, particularly on Sertoli cells (SCs), remain unknown. More recently, leptin has shown the ability to modulate mitochondrial dynamics, biogenesis and functioning in several cellular systems, including cancer cells. Herein, we studied the effects of leptin in the proliferation and metabolic activity of rat Sertoli cells (rSCs). We also evaluated the effects of leptin in mitochondria physiology, particularly in the levels of mitochondrial complexes, messenger RNA (mRNA) levels of mitochondrial biogenesis markers and mitochondrial membrane potential. For comparative purposes, and taking in consideration previous results from the group, we also studied the effects of leptin in mRNA levels of mitochondrial biogenesis markers and mitochondrial complexes in human Sertoli cells (hSCs).

Our results suggest that leptin modulates the metabolic activity and mitochondrial function in rSCs after exposure to a concentration of 50 ng/mL, which mimics a concentration found in morbidly obese men. These findings suggest that high concentrations of leptin, such as those found in morbidly obese individuals, modulate mitochondrial function in rSCs, which could represent a novel mechanism through which leptin contributes to obesity-induced subfertility or infertility in males. Interestingly, leptin exposure had no effect in several aspects of mitochondria physiology, such as mRNA levels of mitochondrial biogenesis markers and levels of mitochondrial complexes which further indicates that leptin seems to affect mitochondrial function. In hSCs, the mRNA levels of Sirtuin 1 (*SIRT1*) presented changes in the group treated with 50 ng/mL of leptin. Protein levels of mitochondrial complex II presented changes in the groups treated with 5 and 50 ng/mL of leptin while in rSCs no differences were observed. Thus, rSCs and hSCs seem to be differently affected by leptin exposure. These differences, particularly in *SIRT1* mRNA levels, are species-dependent and may represent a novel mechanism through which leptin affects the metabolic control of spermatogenesis and thus, with implications in hSCs.

Keywords

Leptin; Obesity; Sertoli cells; Spermatogenesis; Infertility; Mitochondria.

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List of Abbreviations

18S	18S ribosomal RNA
AgRP	Agouti-related peptide
ALCs	Adult Leydig cells
AMPK	AMP-activated protein kinase
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BMI	Body mass index
BSA	Bovine serum albumin
BTB	Blood-testis barrier
CART	Cocaine- and amphetamine-regulated transcript
cDNA	Complementary DNA
DIO	Diet-induced obesity
DMEM:F12	Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FLCs	Fetal Leydig cells
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
HIF-1α	Hypoxia inducible factor 1α
HIFs	Hypoxia inducible factors
HPG	Hypothalamic-pituitary-gonadal
hSCs	Human Sertoli cells
IGF-1	Insulin-like growth factor 1
ITS	Insulin-transferrin-sodium selenite
JAK2	Janus kinase 2
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide
LepR	Leptin receptor
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
MFN2	Mitofusin-2
M-MLV RT	NZY M-MuLV reverse transcriptase

M-PER	Mammalian Protein Extraction Reagent
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ND1	Mitochondrially encoded NADH dehydrogenase 1
NPY	Neuropeptide Y
NRF1	Nuclear respiratory factor 1
OXPHOS	Oxidative phosphorylation
P450SCC	Cytochrome P450 family 11 subfamily A member 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator 1α
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PMCs	Peritubular myoid cells
POMC	Pro-opiomelanocortin
PPAR-γ	Peroxisome proliferator-activated receptor-γ
qPCR	Quantitative PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rSCs	Rat Sertoli cells
RT-PCR	Reverse-transcriptase polymerase chain reaction
s6k1	S6 kinase-beta 1
SCs	Sertoli Cells
SEM	Standard error mean
SHP2	Protein tyrosine phosphatase 2
SIRT1	Sirtuin 1
SOCS3	Suppressor of cytokine signaling 3
SRB	Sulforhodamine B
StAR	Cholesterol transporter steroidogenic acute regulatory protein
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
TFAM	Transcription factor A, mitochondrial
tRNA	Total RNA
ß2M	$ß_2$-microglobulin
$ß2M_{nc}$	Nuclear encoded $ß_2$-microglobulin

I. Introduction

1. Male reproductive system: an overview

The reproductive system is not essential for the survival of the individual; it is, however, required for the survival of the species. It is through the reproductive system that new individuals are born; the species are constantly repopulated and the genetic code is transmitted over generations. In humans, the sexual reproduction is the method used, which has several advantages, namely at the level of variability induced by the combination of progenitor's genes. This variability ensures the evolution of the species throughout time. The reproductive system has some unique features. Unlike any other body systems, it is not fully functional at the time of birth, and it requires the action of sex hormones around the time of puberty to be fully active and ready to perform its purpose. In addition, the gender differences between the male and female reproductive system are clearly observed, a fact that does not occur in the other body systems (Van De Graaff 2001).

The male reproductive system has different structures that can be divided in primary and secondary sex organs. In males, the primary sex organs, also known as gonads, are the testes. They are responsible for the production of spermatozoa and secretion of sex hormones. The secretion of sex hormones is then responsible for the secondary sex organs development. Surrounding the testes is the scrotum, an outpouching of the abdominal wall that protects the testes. The secondary sex organs are structures responsible for the nourishment and storage or transport of the spermatozoa to the exterior or into the female reproductive tract. One of the organs responsible for this transport is the penis. The penis is the male organ used in sexual intercourse and can be divided into three structures: the root linked to the abdominal wall, the body of the penis that corresponds to the major portion of this organ and the glans, also referred as the head of the penis (Clark 2005). There are other secondary sex organs, such as the epididymis, *vas deferens*, ejaculatory ducts and urethra responsible for storage, maturation and transport of the spermatozoa and others responsible for secretion of fluids that are part of the ejaculate, such as seminal vesicles, prostate gland and bulbourethral glands (Figure 1). Sex hormones are also responsible for the development of the secondary sex characteristics, that appear during puberty, such as body hair, deep voice and Adam's apple development (VanPutte, Russo et al. 2010).

The testes are the male gonads, paired ovoid organs that are responsible for the production of spermatozoa and sex hormones. They are suspended in the scrotum by the spermatic cords. Each one is about 4-5 cm long and 2.5 cm in diameter and weighs between 14-18 g in humans (Johnson, Petty et al. 1984). Both testes are covered by two tunics. The

outer tunica is the tunica vaginalis and their visceral layer covers the surface of each testis, except where the testis attaches to the epididymis and spermatic cord. This tunica is a thin closed peritoneal sac that has origin on the peritoneum during testes descent. The parietal layer of the tunica vaginalis covers more tissue than the previous one, extending superiorly onto the distal part of the spermatic cord. The separation between the visceral and parietal layers is filled with fluid, allowing the movement of the testes in the scrotum (Van De Graaff 2001, Rizzo 2009, VanPutte, Russo et al. 2010). The testes also have a tough fibrous outer membrane called tunica albuginea. This tunica has characteristic extensions that move to the inside of each testis dividing it into testicular lobules. Each of these lobules contains long and highly coiled seminiferous tubules that are nearly 80 cm long, if uncoiled in humans. It is inside these tubules, considered the functional unit of the testis, that spermatogenesis occurs (Figure 1). Here, spermatozoa are produced at a rate of about thousands per second. Sertoli cells (SCs), which function as a physical support to germ cells and nourish their development into sperm, form the seminiferous tubules. Between these numerous tubules is the interstitial space, where Leydig cells are placed.

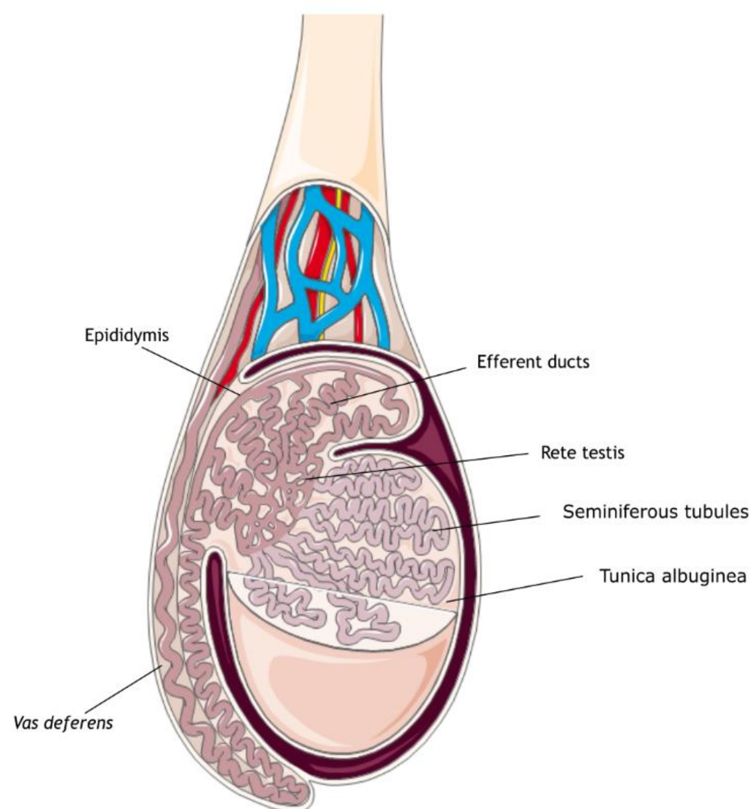

Figure 1: Schematic representation of a human testis. The testis is coated by tunica albuginea, and divided in lobules. The seminiferous tubules are located inside these lobules, highly coiled and organized. The rete testis is responsible for transporting the spermatozoa from the seminiferous tubules into the efferent ducts. From this point forward, spermatozoa enter the epididymis where they go through several processes until they are ready to leave the male reproductive tract into the female reproductive tract.

1.1 Functional organization of the testes

The testes are responsible for the production of male gametes and male sex hormones, which are processes known as spermatogenesis and steroidogenesis, respectively. These two distinct processes take place in two morphologically and functionally different compartments: the seminiferous tubular compartment and the interstitial compartment. Despite being anatomically and functionally different, these two compartments function closely to one another and they are both required to achieve the correct parameters in sperm quality and production.

The tubular compartment is composed by the seminiferous tubules. It is responsible for 60 to 80% of the testicular volume and contains the germ cells, SCs and the peritubular myoid cells (PMCs). Sertoli and germ cells are organized in a highly-polarized system to efficiently support the spermatogenesis. Adjacent SCs form tight junctions with each other, providing an immune-privileged microenvironment suitable to germ cells development. Tight junctions formed between adjacent SCs establish the blood-testis barrier (BTB), responsible for controlling the movement of nutrients in the seminiferous tubules (Wong and Cheng 2005). However, there are other types of cooperation used between adjacent SCs to strengthen the BTB, such as ectoplasmic specializations and desmosomes (Mruk and Cheng 2004, Lie, Cheng et al. 2011). This barrier has the function of "gate", preventing solutes and large molecules from reaching the germ cells and the function of "fence", restricting the movement of proteins and lipids between adluminal and basal compartments. BTB also divides the seminiferous tubules in basal and adluminal compartments. Spermatogonia and pre-meiotic spermatocytes are present in the basal compartment whereas meiotic spermatocytes, spermatids and spermatozoa reside in the adluminal compartment, showing distinct polarity depending on their location (Pelletier 2011, Mruk and Cheng 2015). In addition, Sertoli cell nuclei and Golgi complexes are also found in the basal compartment where early phagosomes and early processes involved in spermatids development are all confined to the adluminal compartment. However, the most obvious form of cell polarity present in the testes is observed during the development of spermatids, where the heads of spermatids point towards the basal compartment while the tails point towards the adluminal compartment (Gao and Cheng 2016, Gao, Xiao et al. 2016). The testis is divided by a septum of connective tissue into about 250-300 lobules, each one containing 1-3 convoluted seminiferous tubules. Generally, there are about 600 seminiferous tubules present in the human testis and each one has an average length of 30-80 cm which varies according to whether they are uncoiled. The total length of the seminiferous tubules is, on average, 300 meters per testis and 600 meters per man (Xiao, Mruk et al. 2014, Griswold 2016).

Surrounding the seminiferous tubules, in the interstitial compartment, are the PMCs which have several functions. In humans, three or four layers of PMCs surround the seminiferous tubules while in mice, a single layer of PMCs is present (Gardner and Holyoke

1964). In adult testis, PMCs mediate the contraction of the seminiferous tubules and, during testis development and adulthood, they work together with SCs to deposit the basement membrane (composed by laminin, collagen IV and fibronectin) that surrounds the seminiferous tubules. This is a critical interaction to ensure a correct spermatogenesis and architecture of the seminiferous tubules (Skinner, Tung et al. 1985, Bichoualne, Thiébot et al. 1994, Maekawa, Kamimura et al. 1996, Verhoeven, Hoeben et al. 2000). There are other functions attributed to PMCs. In rats, these cells where shown to be an important part of the barrier function, restricting the entry of substances into the seminiferous tubules (Dym and Fawcett 1970). The interstitial compartment is located between the seminiferous tubules and is filled with Leydig cells, the major component of this compartment. In fact, there are two populations of Leydig cells, the fetal Leydig cells (FLCs) and the adult Leydig cells (ALCs) (O'Shaughnessy, Baker et al. 2006). After birth, FLCs start degenerating and it is not yet clear if they give rise to ALCs. On the other hand, ALCs derive from Leydig stem cells, capable of self-renewal. These cells develop into Leydig progenitor cells, which express a number of factors such as luteinizing hormone receptors and 3ß-hydroxysteroid dehydrogenase (Haider 2004). Further differentiation occurs into adult cells that no longer proliferate. In the presence of luteinizing hormone (LH), these cells produce testosterone that is fundamental for the establishment and maintenance of the secondary sex characteristics and the continuation of spermatogenesis (Walker 2011, Smith and Walker 2014). There are other cell types present in the interstitium, namely the immune cells (macrophages, T-cells, dendritic cells), where they respond according to the stimuli received (Perez, Theas et al. 2013). From this group, macrophages are the most abundant in the interstitium, corresponding to approximately 25% of the interstitial cells present in the adult rodent testis (Niemi, Sharpe et al. 1986). Several studies have shown that there is cross-talk between immune cells and spermatogonia, where the number of spermatogonia declines after ablation of macrophages (DeFalco, Potter et al. 2015). These also establish cell junctions with Leydig cells, to facilitate an eventual response.

1.2 Sertoli cells

SCs are the somatic cells present in the seminiferous epithelium that support and nourish the developing germ cells. They are highly polarized cells that extend upwards from the basement membrane of the germinal epithelium to the lumen of the seminiferous tubules in a direct interaction with the developing germ cells (Mruk and Cheng 2004). SCs are irregularly shaped, columnar cells with a characteristic oval nucleus with a dark nucleolus. They have a large surface area which is correlated with the number of germ cells that they can support (Vogl, Vaid et al. 2008). This characteristic is also important for germ cell movement during spermatogenesis. First described in 1865 by Enrico Sertoli, who used the term “mother cells” to describe them, SCs are known as the “nurse cells” for their role in

providing structural and nutritional support for germ cells development (França, Hess et al. 2016). Additionally, SCs also have apoptosis functions, are involved in the establishment of the BTB, secrete the seminiferous tubular fluid that assists in the transport of mature spermatozoa into the epididymis and secrete factors such as inhibin that are involved in the hormonal control of spermatogenesis (França, Hess et al. 2016). The number of SCs is heavily tied with the fertility of an individual as each SC has a limit on the number of germ cells that can support. Since these cells become terminally differentiated during puberty, a lower number of SCs implicates a lower number of supported germ cells, which translates into lower levels of daily sperm production with direct consequences in male fertility (Orth, Gunsalus et al. 1988, Sharpe, McKinnell et al. 2003).

As discussed before, the tight junctions formed between adjacent SCs establish the BTB that controls the microenvironment inside the seminiferous tubules. BTB has two main functions: (1) act as a “gate” and a “fence” to restrict the paracellular flow of substances from the basal to the adluminal compartment. The blood vessels and lymphatic vessels are located in the interstitium between the seminiferous tubules which confers to SCs the task of regulating the entry of nutrients and important molecules, such as hormones, into the adluminal compartment where the later stages of spermatogenesis are occurring. However, the BTB also regulates the entry of harmful components that might be present in the blood vessels such as drugs and chemicals. This characteristic selectivity of BTB is crucial for the establishment of a safe microenvironment for the germ cells development. BTB also acts as a “fence”, restricting the movement of proteins and lipids between the basal and adluminal compartments (Wong and Cheng 2005, Cheng and Mruk 2012); (2) creating an immunological barrier, to prevent the immunological response of anti-sperm antibodies that would attack the developing germ cells, leading to male infertility. The BTB sequesters germ-cell specific antigens that surge during meiosis and spermiogenesis, preventing an immunological response. Furthermore, several studies have shown that SCs also play a role in the maintenance of the testis as an immune-privileged organ by secreting immunosuppressive molecules (Su, Mruk et al. 2011, Mruk and Cheng 2015).

SCs possess a Warburg-like metabolism, prioritizing glycolysis over oxidative metabolism which is a less effective pathway for adenosine triphosphate (ATP) production. Furthermore, SCs also sustain a high glycolytic flux, in a similar way to cancer cells, which is a consequence of the metabolic cooperation required between SCs and germ cells for a successful spermatogenesis (Oliveira, Martins et al. 2015). One of the end products of glycolysis, pyruvate, is then converted to lactate which is a crucial factor for germ cells development due to its anti-apoptotic effect in germ cells. Furthermore, lactate is also the major energy source of germ cells (Robinson and Fritz 1981). However, spermatogonia utilize glucose as the major energy substrate while germ cells in further stages of spermatogenesis such as spermatids use lactate for energy production (Mita and Hall 1982, Nakamura, Okinaga et al. 1984). Spermatozoa also utilize glucose/fructose as the major source of energy

illustrating that the substrate required for energy production changes at each stage of spermatogenesis (Bajpai, Gupta et al. 1998). The metabolism of SCs is also highly susceptible to hormones. Follicle-stimulating hormone (FSH) and insulin have well documented effects in the regulation of SCs metabolism, stimulating glucose metabolism and lactate production (Mita, Price et al. 1982, Oliveira, Alves et al. 2012). SCs metabolism and its regulation is one of the most important processes for a normal spermatogenesis and male fertility. However, a grey area of knowledge surrounding SCs metabolism still exists which when undisclosed could contribute to improve several infertility cases.

2. Spermatogenesis

Spermatogenesis is a multi-step process that produces spermatozoa in the seminiferous tubules determining male fertility (O'Shaughnessy 2014). Beginning at puberty and during the reproductive life of a fertile men, more than 40 million spermatozoa are produced every day (Cheng and Mruk 2013). This process is controlled by several endocrine and other regulatory factors and its duration depends on the species with a duration of 40 to 50 days in rodents and nearly 80 days in humans (Sharpe 1994). The final objective of spermatogenesis is the development of mature spermatozoa with half the number of chromosomes (haploid), from the immature germ cells, spermatogonia (diploid). Immature germ cells undergo several processes including mitosis, meiosis and differentiation to give rise to mature spermatozoa (Rato, Alves et al. 2012, Alves, Rato et al. 2013). This process occurs in the seminiferous tubules, the functional unities of the testis, through a close association of germ cells with the somatic cells, SCs (Walker and Cheng 2005, Rato, Alves et al. 2012). The tight junctions formed between adjacent SCs constitute the BTB that divides the seminiferous epithelium into two compartments: the basal compartment where spermatogonia and pre-meiotic spermatocytes are present and the adluminal compartment where meiotic spermatocytes, spermatids and spermatozoa reside. (Cheng, Wong et al. 2010). SCs are also responsible for the germ cells movement towards the lumen where mature spermatozoa are released. Spermatogenesis can be divided in four different phases: mitosis, meiosis, spermiogenesis and spermiation (O'Shaughnessy 2014). These events are a cycle of cellular changes and can be further divided into stages, with different stages per specie. In humans, spermatogenesis occurs during the 6 stages of the seminiferous epithelial cycle while in mice it occurs during the 12 stages of the seminiferous epithelial cycle and in rats during the 14 stages of the seminiferous epithelial cycle (Cheng, Wong et al. 2010). During this process, germ cells are located from the periphery to the center of the seminiferous tubule, according to their degree of maturation (Alves, Rato et al. 2013).

Spermatogonia are the most immature cells in the testis. In the early stages of spermatogenesis, they go under successive mitosis renewing themselves and giving origin to a new cell type. At this stage, they are called spermatogonia type A (Lie, Cheng et al. 2009). These cells are located outside the BTB, continuing their process of self-renewal until death which is the reason why men, unlike women, are fertile throughout life. After several mitoses, spermatogonia type A has three options: it can divide and form another population of spermatogonia (self-renewal), it can undergo apoptosis or it can differentiate into the first committed stem cell type. Intermediate spermatogonia are the first population of cells committed to becoming spermatozoa and give origin to the spermatogonia type B (Mauch and Schoenwolf 2001). These cells are located in the basement membrane of the seminiferous tubules, surrounded by SCs and are the last cells of the line that go under mitosis. They migrate in direction to the lumen of the seminiferous tubule differentiating into the primary spermatocytes (Mauch and Schoenwolf 2001). The factors behind the decisions to follow the path of differentiation instead of self-renewal or to choose to divide meiotically instead of mitotically are still a matter of debate among the scientific community. Primary spermatocytes undergo a meiotic division, meiosis I, to yield a pair of secondary spermatocytes. These haploid cells then undergo another meiotic division, meiosis II, originating haploid spermatids (Figure 2) (O'Donnell, Robertson et al. 2001).

At last, spermiogenesis takes place, in which spermatids maturation into mature spermatozoa occurs. During spermiogenesis several morphological changes occur that involve development of the flagellum, chromosomal condensation, formation of the acrosome and elongation of the nucleus (Meistrich, Trostle-Weige et al. 1992, Rato, Alves et al. 2012). In humans, 12 spermatid maturation steps are described (Weinbauer, Luetjens et al. 2010). SCs are crucial in this phase, as the adhesive contacts and ectoplasmic junctional specializations between spermatids and SCs are shattered which culminates with the release of spermatozoa into the lumen of the seminiferous tubule, in a process called spermiation (Griswold 1998, Hai, Hou et al. 2014). At this point, spermatozoa are fully differentiated cells; however, they are not yet motile and capable of fertilizing the oocyte. The non-motile spermatozoa are transported to the epididymis in testicular fluid secreted by SCs, on which they acquire motility, in a final step of spermatozoa maturation. The ability to fertilize the egg is acquired during the last stages of spermatogenesis, along the epidydimal duct and during the transit inside the female reproductive tract. These series of biochemical changes in which spermatozoa become functional are referred as capacitation (Figure 2) (Dun, Aitken et al. 2012, Dacheux and Dacheux 2014).

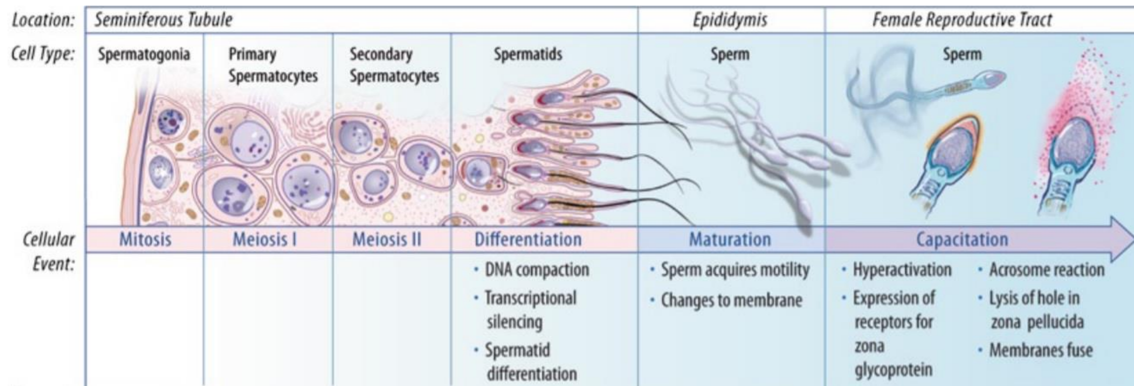

Figure 2: Schematic representation of major events in spermatogenesis. Spermatogonia go through successive mitosis until they differentiate into primary spermatocytes. After the first meiotic division, secondary spermatocytes are formed. Meiosis II follows, which yields haploid spermatids. The spermatids then go through spermiogenesis (differentiation) followed by spermiation, in which they are released into the lumen of the seminiferous tubule. Afterwards, they go through the final steps of maturation in the epididymis, becoming motile. The final steps are achieved in the female reproductive tract, where they go through several biochemical changes, a process known as capacitation. Adapted from (Sharma and Agarwal 2011).

2.1 Hormonal regulation of spermatogenesis

Spermatogenesis is a tightly regulated process due to the complexity and the number of the events involved. A single malfunction in one of the many steps that are part of spermatogenesis can compromise the entire process. Beyond the need to maintain proper growth and differentiation of germ cells, somatic cells proliferation and functionality is also required for a successful spermatogenesis. The hypothalamic-pituitary-gonadal (HPG) axis is responsible for this regulation, through the actions of two endocrine factors, LH and FSH (Ramaswamy and Weinbauer 2014). Within this axis, neurons of the hypothalamus produce gonadotropin releasing hormone (GnRH) in a characteristic pulsatile way (Ramaswamy and Weinbauer 2014). This pulse is susceptible to the metabolic status of body and in a fasting state, GnRH secretion is suppressed which decreases the levels of LH and testosterone compromising male reproductive function if longer periods of fasting occur frequently (Trumble, Brindle et al. 2010). GnRH travels to the anterior pituitary and stimulates the synthesis and release of LH and FSH in the gonadotrophs, the cells that express GnRH receptors. These endocrine factors then act at a testicular level to regulate spermatogenesis (Rato, Alves et al. 2012, Alves, Rato et al. 2013). LH binds to receptors on the surface of Leydig cells and stimulates testosterone production, a sex steroid hormone that diffuses into the seminiferous tubules. Within the seminiferous tubules, SCs are the only cells that possess receptors for testosterone and FSH (Walker and Cheng 2005). FSH is crucial to stimulate spermatogenesis since its involved in the maintenance of testicular size and in the regulation of spermatogonia proliferation and differentiation (Dym 1994, Walker and Cheng 2005). Synergistically, testosterone and FSH stimulate SCs to secrete paracrine agents, such as

androgen-binding protein, that modulate spermatogenesis (Ritzen, Hagenas et al. 1975, Hall, Conti et al. 1990). A complex interaction between the hypothalamus and the testes regulates gonadotropins synthesis and release (Figure 3). On one side, hypothalamus through GnRH production stimulates LH and FSH production; on the other the testes establish a negative feedback mechanism with the pituitary, through the production of sex steroid hormones and inhibin (Tilbrook and Clarke 2001). Several autocrine and paracrine factors are also reported to be involved in local mechanisms that modulate spermatogenesis (Le Magueresse, Pineau et al. 1988, Nehar, Mauduit et al. 1998, Huleihel and Lunenfeld 2002). Inhibin, secreted by SCs, can act on the anterior pituitary decreasing the FSH levels (O'Connor and De Kretser 2004) while its counterpart, activin is involved in SCs proliferation (Nicholls, Stanton et al. 2012). Testosterone, produced by Leydig cells, inhibits LH production through two distinct mechanisms: it acts on the hypothalamus diminishing the frequency of the GnRH pulsatile release which ultimately decreases the secretion of gonadotropins in the pituitary; or acts directly in the pituitary decreasing the secretion of LH (Figure 3) (Widmaier, Raff et al. 2011). Testosterone has a crucial role in male reproductive function, as its absence prevents germ cells to go beyond meiosis, prevents the release of mature spermatozoa and compromises the establishment of the BTB (Walker 2010). The disturbance of one of these steps compromises spermatogenesis and consequently male fertility.

Spermatogenesis regulation is not summed up to androgens and gonadotropins. In fact, estrogens, insulin-like growth factor 1 (IGF-1) and growth hormone have showed distinct functions with implications in spermatogenesis. Estrogens, usually referred as the female sex hormone, are reported to have an essential role regulating the HPG axis, indirectly modulating LH and testosterone equilibrium through a feedback loop (O'Donnell, Robertson et al. 2001). IGF-1, synthetized in the testes by SCs, has also shown to be capable of stimulate the proliferation of Leydig cell precursors and spermatid maturation (Itoh, Nanbu et al. 1994). Additionally, despite usually being referred as being involved in other processes, growth hormone also has an important role in reproductive function. Growth hormone plays a role in gonadal steroidogenesis and spermatogenesis either directly at a gonadal level or indirectly via IGF-1. In fact, growth hormone regulates Leydig cells steroidogenesis through regulation of IGF-1 production (Spiteri-Grech and Nieschlag 1992, Hull and Harvey 2000). Despite a lot of knowledge acquired in the last decades about the hormones and processes involved in spermatogenesis, there is still much to be done. The complex signaling and hormonal network behind the control of spermatogenesis still needs to be deciphered to better understand the mechanisms behind male fertility and to understand which part of this endless chain of biochemical events is comprised in infertility cases.

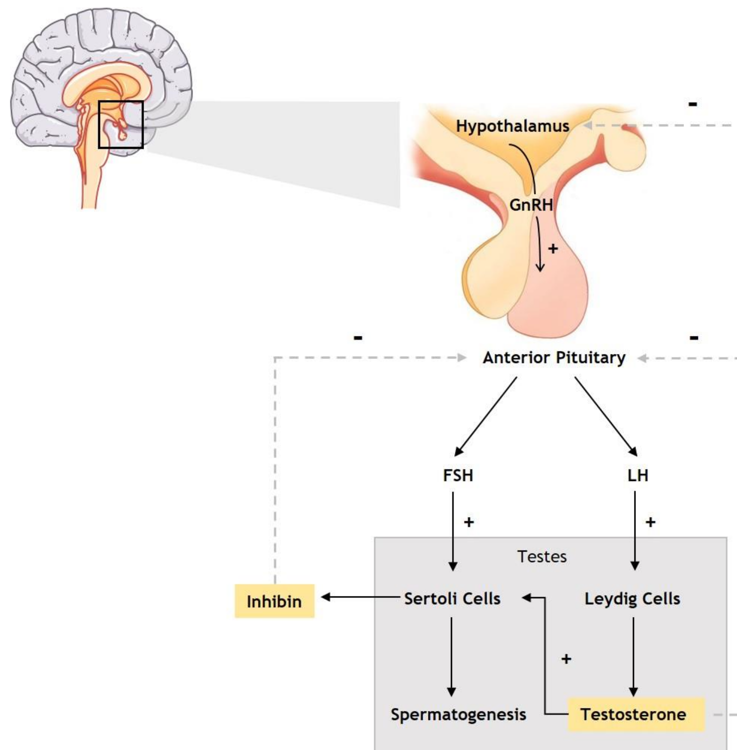

Figure 3: Hormonal regulation of male reproductive function. GnRH is synthetized in the hypothalamus which in turn stimulates the anterior pituitary to produce LH and FSH. LH acts on Leydig cells stimulating testosterone production while FSH acts on Sertoli cells, stimulating spermatogenesis. High testosterone levels inhibit the release of GnRH by hypothalamus and LH by anterior pituitary. Inhibin production by Sertoli cells negatively regulates FSH production by anterior pituitary. Abbreviations: GnRH – gonadotropin releasing hormone, LH – luteinizing hormone, FSH – follicle-stimulating hormone. Legend: + stimulation; - inhibition.

3. Leptin: from discovery to energy control

Leptin was discovered in 1994 by Friedman *et al.*, through positional cloning (Zhang, Proenca et al. 1994). Since then, the knowledge about this adipocyte-derived protein has consistently grown. In brief, leptin is a 16 kDa cytokine encoded by the *LEP* gene, located in chromosome 7q31.3 in humans consisting of three exons separated by two introns, and chromosome 6 in mice (He, Chen et al. 1995, Isse, Ogawa et al. 1995). The majority of its production occurs in adipocytes but it can also be locally produced in other tissues such as stomach, placenta, ovary, mammary gland, immune cells and skeletal muscle (Cioffi, Van Blerkom et al. 1997, Bado, Levasseur et al. 1998, Schubring, Prohaska et al. 1999, Wang, Wang et al. 2011, Wolsk, Mygind et al. 2012). In these tissues, leptin has been implicated in several functions such as regulation of immune responses, reproductive function, and fetal development (Lago, Gomez et al. 2008, Herrid, Palanisamy et al. 2014, Perez-Perez, Sanchez-Jimenez et al. 2015). These evidences indicate that leptin has pleotropic effects in several biological functions and more importantly, that leptin function is generally independent of its

action in the regulation of body weight. After being produced by white adipose tissue, leptin enters the bloodstream and circulates in a concentration that is associated with the amount of adipose tissue depots on an individual (Frederich, Hamann et al. 1995, Maffei, Halaas et al. 1995). Therefore, leptin levels indicate body fat reserves. This information is then communicated to specific areas in the brain that regulate energy homeostasis (Rosenbaum and Leibel 2014). Higher circulating leptin levels suggest a higher number of adipose tissue depots which triggers a response to reduce food intake and to promote energy consumption. In contrast, a decrease in leptin circulating levels triggers an opposite response, promoting food intake and reducing energy consumption (Hamann and Matthaei 1996, Kolaczynski, Ohannesian et al. 1996). This mechanism is often dysregulated in several metabolic diseases such as obesity. In those cases, leptin resistance occurs where higher levels of circulating leptin are present but do not trigger the expected response in order to reduce food intake and promote energy expenditure (Jung and Kim 2013, Sainz, Barrenetxe et al. 2015). Thus, since its discovery, leptin has emerged as a key control point for energy homeostasis and its relevance in health and disease has been in spotlight for more than two decades. In the last years, the effects of leptin in male reproductive function gained attention and novel molecular mechanisms showing how it acts through the male reproductive tract have been identified.

3.1 Leptin as a satiety hormone

Leptin exerts its physiological effects through specific receptors. First identified in 1995 (Tartaglia, Dembski et al. 1995), the leptin receptor (LepR) was isolated from mouse choroid plexus and due to strong similarities in the extracellular ligand-binding domain with the gp130 signal-transducing subunit is considered part of the large family of class I cytokine receptors. This receptor is encoded by the *LEPR* gene located in the chromosome 1 and chromosome 4 in humans and mice, respectively. Activated via ligand induced conformational changes, this receptor forms homodimers even when leptin is not present (Devos, Guisez et al. 1997). Several isoforms of this receptor are known (LepRa-f), all products of the *LEPR* gene, resulting of alternative messenger RNA (mRNA) splicing or post translational modifications (Lee, Proenca et al. 1996). These isoforms have identical extracellular domains consisting of over 800 amino acids; however, their intracellular domains terminate at different lengths that are characteristic of each isoform (Gorska, Popko et al. 2010).

Secreted isoforms are characterized by only possessing an extracellular domain unlike the other isoforms. This can result of alternative mRNA splicing or proteolytic cleavage products of membrane-bound isoforms of LepR. Discovered by Sinha *et al.*, this isoform is soluble and functions as the main binding protein for leptin circulating in the bloodstream,

modulating its bioavailability and the cellular effects of leptin itself (Sinha, Opentanova et al. 1996, Lammert, Kiess et al. 2001). Unlike what happens in mice, the human secreted isoform (LepRe) is exclusively generated through proteolytic cleavage of membrane-bound isoforms of LepR, a process referred as shedding, thus being an excellent indicator of the number of membrane-anchored leptin receptors (Maamra, Bidlingmaier et al. 2001). In normal conditions this process occurs inherently, however several studies have shown that it can be induced by different kinds of stimuli such as lipotoxicity and apoptosis (Unger 2003, Schaab, Kausch et al. 2012). Soluble isoforms can have either antagonistic or agonistic effects although the former prevails (Di Marco, Gloaguen et al. 1997, Hardy, Owczarek et al. 2001, Chalaris, Garbers et al. 2011). LepRe effect is still a matter of debate, however it has been shown that an antagonistic effect is present (Schaab, Kausch et al. 2012). Higher concentrations of LepRe over leptin have a prevailing inhibitory effect on leptin signal transduction, nullifying a crucial step of leptin-mediated activation, the signal transducer and activator of transcription 3 (STAT3) activation thus neutralizing the anorexigenic effects of leptin in rats (Zhang and Scarpace 2009). Moreover, several other mechanisms through which LepRe promotes leptin resistance have been identified including inhibition of leptin transport through the blood-brain barrier (BBB) (Tu, Kastin et al. 2008). These antagonistic effects are also present in a state of negative energy balance, such as anorexia nervosa or malnutrition, where LepRe is found in higher concentrations when compared with leptin (Krizova, Papezova et al. 2002, Stein, Vasquez-Garibay et al. 2006).

Short isoforms (LepRa, LepRc, LepRd, LepRf) are very similar between them and their role is not yet very clear. They possess a similar extracellular domain and a similar transmembrane domain consisting of 34 amino acids. Their intracellular domain is also similar, sharing the first 29 amino acids, and consisting of 32-40 amino acids in total (Wada, Hirako et al. 2014). Due to lack of intrinsic tyrosine kinase activity, LepR binds to cytoplasmic kinases, specifically Janus kinase 2 (JAK2) (Ghilardi and Skoda 1997). The first 29 amino acids contain a constant box 1 motif and a JAK2. Although these isoforms are also able to recruit JAKs and activate certain signaling cascades, they lack the box 2 motif to be able to induce the pivotal STAT. More recently, studies highlight for their involvement in leptin signaling cascades through MAP kinase signaling pathways (Akasaka, Tsunoda et al. 2010). Other possibility is their involvement in leptin transport across the BBB and leptin clearance (Hileman, Tornoe et al. 2000, Hileman, Pierroz et al. 2002).

LepRb, also known as long isoform of LepR, is the fully functional isoform of leptin receptor. The major difference between LepRb and the short isoforms resides in the intracellular domain, formed by over 300 amino acids (Gorska, Popko et al. 2010). Similar to what happens in short isoforms, this domain contains a box 1 motif and a JAK2, however it also contains two box 2 motifs that are crucial to induce the STAT signaling pathway, characteristic of leptin signaling (Allison and Myers 2014). This isoform is essential to the

correct signaling of leptin as seen in animal models that lack this receptor, such as db/db mice. Homozygous for the LepR spontaneous mutation and thus lacking the LepRb isoform, phenotypically these animals are hyperleptinemic, hyperphagic, obese, leptin resistant and have reproductive dysfunction (Chen, Charlat et al. 1996). These animals also display a phenotype similar to db3J/db3J mice, a model that is null for all known isoforms of the LepR. Interestingly, expression in these mice of a LepRb transgene induces a depletion of body weight, hyperphagia, glucose intolerance and interestingly, it restored male fertility (Kowalski, Liu et al. 2001). However, mutations in the *LEPR* and *LEP* gene are extremely rare in humans. In male reproductive tract, LepR has already been identified in the testes, epididymis and seminiferous tubules in several species (Rago, Aquila et al. 2009). In humans, LepR was already identified in somatic cells, germ cells and spermatozoa although its function and involvement in reproductive processes such as spermatogenesis and steroidogenesis is not yet enlightened (Jope, Lammert et al. 2003, Ishikawa, Fujioka et al. 2007, Martins, Moreira et al. 2015). However, several findings point towards a significant role of leptin in these cells underpinning the importance of further scientific research in this specific field.

3.1.1 Leptin and LepR expression in testis

The first demonstrations of LepR expression in murine testis, specifically in sperm and Leydig cells, appeared soon after leptin itself was discovered (Hoggard, Mercer et al. 1997). After these findings, several studies have shown that LepR expression in testis is a shared trait among different species (Rago, Aquila et al. 2009). Interestingly, LepR isoforms present different expressions levels per tissue. For example, LepRb, the fully functional signaling isoform of LepR, is found mainly in the hypothalamus, while LepRa, a short isoform with a role that is not yet clear, is found in peripheral tissues of most species studied, including the testis (Margetic, Gazzola et al. 2002). In fact, some studies report that LepRb is not found at all in testis while others report that LepR in testis is functional and JAK/STAT pathways are involved in leptin signaling (Hoggard, Mercer et al. 1997, Yuan, Huang et al. 2014). This could be due to the fact that LepR activation involves cell-specific signal transduction pathways. Moreover, LepR short isoforms still have signaling potential and are still able to recruit JAKs and activate certain signaling cascades which could explain leptin signaling occurring without the functional isoform of LepR. However, there is very little information about LepR short isoforms and the signaling cascades involved which is an obstacle when trying to understand the possible leptin signaling implications in the testis related to these isoforms.

LepR connection with Leydig cells started to be in the spotlight as soon as the inhibitory effect of leptin in testosterone production was established. LepR has been already identified in Leydig cells in several species including humans (Caprio, Isidori et al. 1999,

Caprio, Fabbrini et al. 2003). Leydig cells, located in seminiferous tubules, are responsible for the production of the primary male sex hormone, testosterone. Leptin has shown to inhibit testosterone secretion at a testicular level in rat testis, depending on the state of sexual maturation (Tena-Sempere, Pinilla et al. 1999). Posterior studies have shown that LepR expression is characteristic of mature Leydig cells (Caprio, Fabbrini et al. 2003). Furthermore, increasing concentrations of leptin administrated in rodent Leydig cells led to a dose-dependent inhibition of human chorionic gonadotropin-stimulated testosterone production, indicating that Leydig cells are the likely target for leptin-mediated inhibition of testosterone production (Caprio, Isidori et al. 1999). Additionally, in men, leptin levels are inversely correlated with testosterone levels which strongly suggests that leptin can modulate testicular steroidogenesis, probably through Leydig cells (Luukkaa, Pesonen et al. 1998). Recent studies have also shown that hyperleptinemia downregulates cAMP-dependent activations of the cholesterol transporter steroidogenic acute regulatory protein (StAR) and of the rate-limiting steroidogenic enzyme cytochrome P450 family 11 subfamily A member 1 (P450SCC) in Leydig cells. These findings suggest that higher values of leptin found in the obesity condition downregulate the STAT transcriptional activity, leading to lower expression levels of cAMP-dependent steroidogenic genes, involved in several processes such as testosterone production (Landry, Sormany et al. 2017). AMP-activated protein kinase (AMPK) signaling pathway has shown the ability to downregulate Leydig cells steroidogenesis and could be involved in this process (Abdou, Bergeron et al. 2014). Leptin may rather upregulate this pathway that is known to inhibit *StAR* expression, in a condition where higher levels of leptin are present (Christenson, Osborne et al. 2001, Li, Xu et al. 2011). Furthermore, activated STAT transcription factors have also shown to downregulate *StAR* and *Cypa11a1* expression from Leydig cells (Roumaud and Martin 2015). However, further studies are needed to better define how leptin influences and modulates Leydig cells function and possible consequences in male reproductive processes.

Contrary to Leydig cells, there is not much information available relating SCs with leptin. However, LepR was already identified in these cells in rats and more importantly in humans, which indicates a role for leptin signaling that still needs to be unveiled (Tena-Sempere, Manna et al. 2001, Martins, Moreira et al. 2015). Moreover, a recent study has shown that different concentrations of leptin change human SCs (hSCs) acetate production highlighting leptin ability in modulating SCs metabolism, which could have implications in spermatogenesis since these cells are responsible for the nutritional support of that process (Martins, Moreira et al. 2015). Furthermore, several studies have shown that in the ob/ob obese and infertile mice model (mice lacking endogenous leptin), Sertoli and germ cells present a condensed nucleus with higher levels of fragmentation. Spermatogonia, spermatocytes and spermatids are also present in lower levels when compared to controls, which indicates leptin importance for a normal spermatogenesis (Martins, Aguila et al. 2017). Altogether, SCs could be one of the targets of leptin in the testis with further effects in

fertility; however, there is still much to be discovered namely leptin signaling pathways associated in SCs; which pathways are involved and their importance and involvement concerning spermatogenesis.

Similarly to somatic cells, germ cells and spermatozoa also have leptin receptors identified in several species including humans (Jope, Lammert et al. 2003, De Ambrogi, Spinaci et al. 2007, Nikbakht, Mehr et al. 2010, Lange-Consiglio, Corradetti et al. 2016). However, existing studies are contradictory with some reporting the presence of the receptor and others refuting it (Hatami-Baroogh, Razavi et al. 2010). In spermatozoa from pig, leptin might be involved in capacitation, as LepR is located in the acrosome region. In fact, in a study by Aquila *et al*. both capacitation indexes and acrosin activity were enhanced by leptin. Furthermore, they observed that leptin triggers STAT3 and induces the activation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and MAPK pathways and B-cell lymphoma 2, an anti-apoptotic protein (Aquila, Rago et al. 2008). Leptin was able to improve sperm cryopreservation through activation of certain antioxidant enzymes (Fontoura, Mello et al. 2017). These findings suggest that leptin has a role in sperm capacitation and survival, however the molecular mechanisms responsible for this action are still undisclosed.

3.2 Overview of leptin signaling and its relevance in male reproductive tract

Leptin signaling is mainly mediated by the long isoform of leptin receptor, LepRb. This isoform is expressed in specific areas of the brain involved in the regulation of feeding and energy expenditure, such as the neurons located in the nuclei of hypothalamus (van Swieten, Pandit et al. 2014). After being produced by adipocytes, leptin circulates in the bloodstream and bounds to LepRe (Lammert, Kiess et al. 2001). Then, it surpasses the BBB through mechanisms not yet enlightened. Once in the hypothalamus, leptin binds to the extracellular domain of LepRb in the nuclei inhibiting hunger, counteracting orexigenic factors such as neuropeptide Y (NPY) and agouti-related peptide (AgRP) and enhancing the actions of the anorexigenic peptides α-MSH/pro-opiomelanocortin (α-MSH/POMC) and cocaine- and amphetamine-regulated transcript (CART) (Schwartz, Woods et al. 2000, Murphy 2005). Leptin binding causes a conformational change in the receptor that in turn enables the activation of JAK2, which phosphorylates other tyrosine residues located in the LepRb-JAK2 complex triggering several downstream signaling pathways (Couturier and Jockers 2003). So far three tyrosine residues where identified located in the intracellular domain of LepRb, Tyr_{985}, Tyr_{1077} and Tyr_{1138}. Phosphorylation of each one of these residues sets in motion a different set of downstream signaling proteins.

Tyr_{1138} phosphorylation recruits STAT3, that is later phosphorylated by JAK2. After this, STAT3 dimerizes and translocates to the cell nucleus, where it acts as a transcription

factor of certain genes such as suppressor of cytokine signaling 3 (*SOCS3)*, an inhibitor of LepR signaling. SOCS3 is a pivotal part of the negative feedback loop mechanism that regulates leptin signaling, binding to Tyr_{985} and inhibiting JAK2, restraining its activity (Figure 4) (Babon and Nicola 2012). In fact, SOCS3 hypothalamic overexpression has been suggested as one possible mechanism for the development of leptin resistance, a hallmark of obesity (Lubis, Widia et al. 2008). In adipose tissue, this seems to be the primary autoregulatory mechanism between leptin and its receptor through which leptin levels are kept in normal levels. Evidence of this mechanism has also been found in the prostate, however concerning the male reproductive tract, there are no reports that this autoregulatory mechanism occurs in the testis (Colli, Silveira Cavalcante et al. 2011). Phosphorylated STAT3 also helps in the transcriptional regulation of neuropeptides mediated by leptin and involved in appetite regulation, such as POMC, AgRP and NPY. Specifically, LepRb→STAT3 has shown to regulate anorexigenic POMC levels in hypothalamus, probably one of the key functions through which STAT3 regulates energy balance. However, in the orexigenic neuropeptides such as AgRP and NPY, STAT3 has not shown the same effects suggesting that LepRb→STAT3 signaling is insufficient to cause leptin-mediated suppression of NPY and AgRP and thus, other signaling pathways are suggested to be involved (Bates, Stearns et al. 2003).

Tyr_{1077} phosphorylation promotes the recruitment and transcriptional activation of signal transducer and activator of transcription 5 (STAT5) by JAK2, although it is suggested that Tyr_{1138} is also necessary to STAT5 activation (Gong, Ishida-Takahashi et al. 2007). STAT5 signaling has been shown to be involved in the regulation of energy homeostasis. In fact, mice with STAT5 deletion in the brain developed severe obesity accompanied with hyperphagia, hyperleptinemia and insulin resistance with alterations in the regulation of energy expenditure (Lee, Muenzberg et al. 2008). Additionally, several studies have associated STAT5 with the reproductive function. In fact, concerning female reproduction, exogenous prolactin administration showed to induce STAT5 activation in areas of the brain related with fertility (Cave, Norman et al. 2005). Furthermore, STAT5 has shown to have an important role in the sexual dimorphism that exists in the liver (Holloway, Cui et al. 2007). However, a study with three different mice knockout populations (LepR specific deletion of STAT3, STAT5 or both STAT3 and STAT5) revealed that despite increased body weight in STAT3 and STAT3 together with STAT5 knockout mice, fertility parameters and puberty onset presented no differences relatively to the control group (Singireddy, Inglis et al. 2013). These findings indicate that LepRb→STAT3 and LepRb→STAT5 are not crucial pathways regulating leptin role in fertility, suggesting the involvement of other pathways mediating this effect. Further studies are needed to enlighten the exact molecular pathways in leptin signaling concerning human reproduction.

Finally, protein tyrosine phosphatase 2 (SHP2) is recruited to phosphorylated Tyr_{985}. Activation of hypothalamic extracellular signal-regulated kinase (ERK) ensues, mediating

some of the physiological effects of leptin action (Figure 4) (Zhang, Chapeau et al. 2004). ERK signaling involvement in energy homeostasis was first suggested when ERK activation was observed in the arcuate nucleus (ARC) and other areas of the brain during fasting, a state reversed after food intake (Ueyama, Morikawa et al. 2004). Pharmacological blockade of this signaling results in an inverse phenotype compared with normal leptin effects, reversing the weight-reduce effects of leptin, cementing hypothalamic ERK as crucial in the control of energy homeostasis (Rahmouni, Sigmund et al. 2009). Besides Tyr_{985} phosphorylation, another mechanism seems to be involved in leptin-induced ERK activation, probably through some of the short isoforms of LepR whose main function and mode of action are still undisclosed (Bjorbaek, Uotani et al. 1997). Initially suggested as an inhibitor of leptin signaling, SHP2 has shown to be essential for a correct leptin signal transduction. Indeed, deletion of SHP2 from POMC neurons and forebrain promotes early onset obesity and turns the body prone to diet-induced obesity (DIO) (Banno, Zimmer et al. 2010). These data suggest that LepRb→SHP2→ERK signaling is crucial in the maintenance of energy homeostasis, contrary to the first concepts and suggestions. However, SHP2 is involved in several signaling pathways, such as JAK/STAT signaling pathways and the insulin receptor signaling pathway, among others, which makes very difficult to assess the true role of SHP2 in leptin signaling (Xu, Schwab et al. 2014). Besides the involvement in ERK signaling, Tyr_{985} also serves as a binding site for SOCS3, involved in the negative feedback loop that suppresses leptin signaling (Bjorbak, Lavery et al. 2000).

Leptin signaling mediated through the long isoform of LepR also involves other signaling pathways, where leptin can inhibit them such as AMPK signaling or activate them such as PI3K-AKT and mammalian target of rapamycin (mTOR) signaling. During the last two decades, the knowledge about leptin signaling has deepen significantly, particularly its relevance for human health and how it can mediate the deleterious effects caused by some diseases, such as metabolic disorders. In male reproductive system, signaling pathways associated with leptin signaling are known as pivotal to determine male fertility, evidencing the role of leptin in male reproduction.

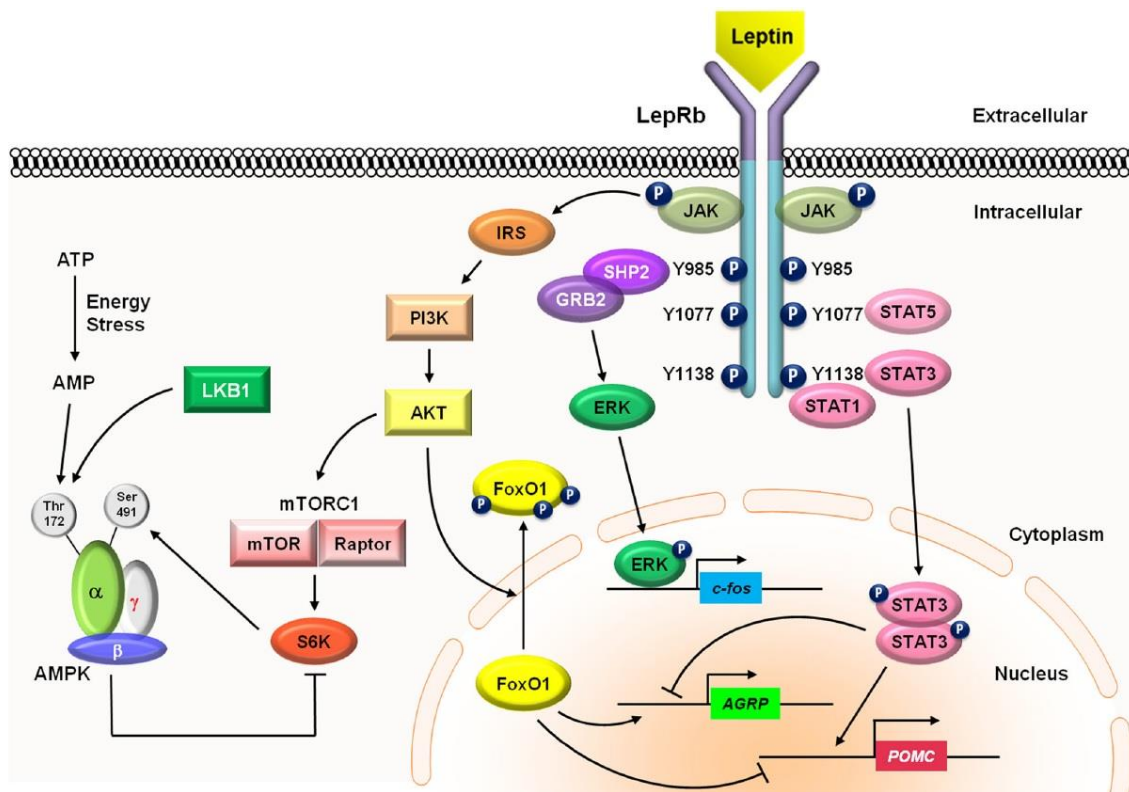

Figure 4: Signaling pathways of leptin. Leptin binds to LepRb receptor causing a conformational change in the receptor, activating JAK2, which phosphorylates other tyrosine residues located in the LepRb-JAK2 complex, triggering several downstream signaling pathways. Leptin signaling through LepRb also involves other signaling pathways, as depicted in the figure. Abbreviations: AgRP - agouti-related protein, JAK -Janus kinase, POMC – proopiomelanocortin, SOCS3 - suppressor of cytokine signaling 3, STAT - signal transducer and activator of transcription. Adapted from (Kwon, Kim et al. 2016).

4. Leptin – devil or angel in obesity

The discovery of leptin in the early 90’s, brought high expectations and attention due to its expected role in solving a long-time problem: the obesity epidemic, already a matter of concern at that time. The ob/ob mice model was critical for leptin discovery. Hyperphagic with low locomotor activity and infertile, they are an excellent model for the study of obesity. After its discovery, leptin administration started to be used as a therapy for obese people but the results obtained were underwhelming (Heymsfield, Greenberg et al. 1999, Hukshorn, Saris et al. 2000). In fact, it was observed that obese people have abnormally higher concentrations of leptin, when compared to lean people in proportion to their increased body fat mass, suggesting that other mechanisms were involved in obesity’s development (Minocci, Savia et al. 2000). Since then, the focus has switched from seeing leptin as a putative therapy agent to understand why in obese individuals, leptin cannot exert the expected effects, despite being present in higher concentrations than in lean individuals.

Nonetheless, in 2014 leptin has gain US Food and Drug Administration (FDA) approval to be used in the treatment of generalized lipodystrophy (Sinha 2014).

Leptin resistance is a concept arisen after studies showing that leptin administration fails to decrease body weight and food intake in obese individuals. Thus, leptin resistance can be defined as the inability of exogeneous leptin to trigger the expect outcomes associated with leptin action. Due to the vast network involved in leptin signaling, it is hard to pinpoint the exact mechanisms that lead to the development of leptin resistance. However, there are several hypotheses for the higher concentrations observed and the diminished leptin response, such as a defective leptin transport across the BBB, a diminished expression of leptin receptors and endoplasmic reticulum (ER) stress (Caro, Kolaczynski et al. 1996, El-Haschimi, Pierroz et al. 2000, Ozcan, Ergin et al. 2009, de Git and Adan 2015).

4.1 Leptin resistance in obesity

Since leptin resistance was first noticed, the major part of studies was focused on the role that negative feedback mechanisms, like SOCS3, have on impairing LepR signaling. Overexpression of these mechanisms can prevent the expected overactivation of leptin signaling pathways in obese individuals, which can lead to the development of leptin resistance. Several signaling pathways and molecules are involved in leptin signaling and some negative feedback mechanisms may be activated. As previous referred, SOCS3 expression is induced by STAT3, which in turn binds to Tyr_{985} switching off leptin signaling (Starr, Willson et al. 1997). Animal models without neuron SOCS3 or haploinsufficient for SOCS3 have a greater response to exogenous leptin and are even resistant to DIO resulting in a greater suppression of appetite and body weight loss (Howard, Cave et al. 2004, Mori, Hanada et al. 2004). Additionally, mice characterized by leptin resistance have increased hypothalamic SOCS3 expression and SOCS3 overexpression blocks leptin signaling in mammalian cell lines (Dunn, Bjornholm et al. 2005). SOCS3 overexpression in anorexigenic neurons also leads to impairment of STAT3 and mTOR signaling, which was related with obesity and leptin resistance (Reed, Unger et al. 2010). These findings solidify the central role of SOCS3 in mediating leptin resistance (Figure 5). However, some studies in mice have contradictory results, showing that inactivation or overexpression of SOCS3 in neurons expressing LepR does not affect the weight gain through a high-fat diet despite protecting against leptin resistance, which highlights the need for further studies to clarify the exact role of SOCS3 on leptin action (Zhang, Zhang et al. 2008, Reed, Unger et al. 2010).

Leptin signaling dysfunction seems to be associated with obesity-associated inflammation through ER stress, which activates inflammatory signaling pathways (Figure 5). ER is responsible for the correct folding of newly made proteins and transport of these

proteins into the Golgi apparatus. However, a failure in folding newly proteins causes an accumulation of unfolded proteins, leading to ER stress, that in turn triggers the unfolded protein response (Hotamisligil 2010). ER stress is well identified as a trademark of insulin resistance and due to the common features shared between the two types of resistance, it was suggested as a link between obesity and leptin resistance (Guerrero-Hernandez, Leon-Aparicio et al. 2014). Overexpression of molecules associated with the unfolded protein response is observed in hypothalamus of DIO mice (Ozcan, Ergin et al. 2009). Furthermore, central administration of an ER stress inducer inhibits leptin anorexigenic effects through the inhibition of STAT3 activation in hypothalamic neurons (Won, Jang et al. 2009). Administration of an ER stress inhibitor also improves leptin sensitivity and diminishes the ratio of weight gain in DIO mice (Zhang, Zhang et al. 2008, Ozcan, Ergin et al. 2009). Inflammatory signaling pathways seem to be involved in linking ER stress with impaired LepR signaling. ER stress induced by a high fat diet seems to be caused by lipotoxicity, due to an accumulation of lipids in hypothalamic neurons (Choi, Kim et al. 2010, Mayer and Belsham 2010, McFadden, Aja et al. 2014). Interestingly, lipotoxicity also seems to cause proteolytic cleavage of the extracellular LepR domain, which can cause a decrease in the availability of leptin receptors, contributing to the development of leptin resistance (Schaab, Kausch et al. 2012). Lately, a link between mitochondria-ER interaction and leptin resistance has been studied. POMC neurons are a key factor in leptin signaling, however in DIO mice, mitochondria-ER interactions in POMC neurons are decreased (Schneeberger, Dietrich et al. 2013). Mitofusin-2 (MFN2) is a mitochondrial membrane protein, involved in mitochondrial fusion and responsible for mediating mitochondrial-ER interactions (Schrepfer and Scorrano 2016). Ablation of MFN2 specifically in POMC neurons has shown a severely decrease in these interactions and resulted in ER stress, leading to the development of leptin resistance. Interestingly, pharmacologically reversion of ER stress has shown an improvement of these conditions. Furthermore, ablation of Mitofusin-1 and MFN2 in AgRP neurons results in alterations in mitochondria size and density without inducing ER stress with improvements in body weight of mice fed with a high-fat diet (Dietrich, Liu et al. 2013). These results establish mitofusins as a major player in the regulation of whole-body energy metabolism by changing and mediating the mitochondrial-ER axis. Altogether, these studies seem to point towards an important role carried out by ER stress in the development of leptin resistance. Further studies are needed to clarify the exact role of ER stress, which can establish the basis for a novel treatment for obesity.

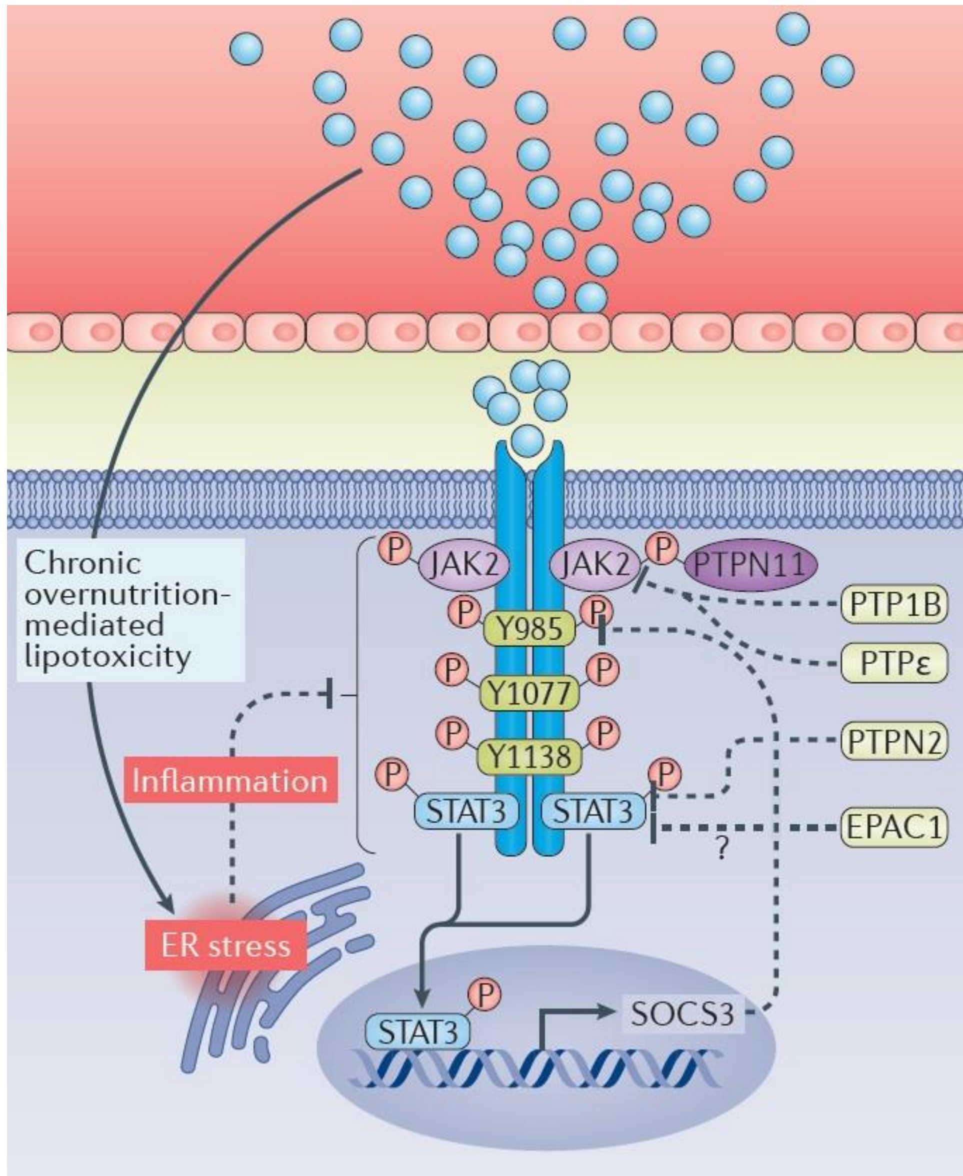

Figure 5: Leptin signaling and the proposed molecular mechanisms behind leptin resistance in obesity. In obese individuals, leptin is present at higher levels. However, a diminished transport across the blood-brain barrier and the action of inhibitory negative feedback mechanisms, like SOCS3 leads to impaired leptin signaling. ER stress also triggers inflammatory responses, which could be involved in the diminished response to leptin that happens in obesity. Leptin signaling pathways are simplified in this image. Solid line – stimulates; Dashed line – inhibits. Abbreviations: SOCS3 – suppressor of cytokine signaling 3, ER – endoplasmic reticulum. Adapted from (Cui, Lopez et al. 2017).

Leptin resistance can be compared with insulin resistance on obesity (Mark 2013). In a similar fashion, leptin resistance seems to be selective, namely leptin seems to exert effects in some systems and at the same time be unable to promote body weight reduction or appetite supression (Mark 2013). For example, leptin can increase blood pressure but cannot suppress food intake, highlighting the role of leptin in the increased blood pressure associated with obesity (Mark, Correia et al. 2002, Correia and Haynes 2004). Thus, it should be highlighted that leptin can exert different functions in different systems when studying the

mechanisms behind leptin resistance and not extrapolate a specific leptin function in a system as a general trait. Besides the similarities between leptin resistance and insulin resistance, leptin resistance also seems to be different relatively to endogenous *vs* exogenous leptin. In fact, intraperitoneal or intracerebroventricular administration of LepR antagonists in DIO mice seems to have the same effect in body weight and food intake when compared to lean mice (Ottaway, Mahbod et al. 2015). However, the administration of the same LepR antagonist in genetic models of obese mice did not produce any results relatively to food intake and body weight changes. These results illustrate that, despite DIO mice present hyperleptinemia and increased body weight, they still retain a similar ability to respond to endogenous leptin compared to lean mice, counteracting the hypothesis that endogenous leptin also contributes to the development and persistence of leptin resistance and obesity. In this case, obesity development seems to occur despite the contribution of endogenous leptin to restore energy balance, which points towards a major role of other mechanisms opposing leptin action, specifically through the LepR, in the development of leptin resistance and obesity. The involvement of other mechanisms when there is an excess of energy available seems to be in play here and the identification of those mechanisms is important to develop a future therapy to counteract co-morbidities associated with obesity.

Leptin transport in gonads seems to be different from the transport in the BBB. In fact, leptin seems to be transported across the BTB through a non-saturable mechanism, which means that BTB does not contribute to the development of leptin resistance (Banks, McLay et al. 1999). However, in individuals with higher concentrations of leptin, for example obese individuals, this could indicate that the microenvironment constituted by SCs, Leydig cells and germ cells, could be exposed to increasing concentrations of leptin, which could have influence in some of the effects observed in sperm count and motility of obese individuals (von Sobbe, Koebnick et al. 2003).

5. Hormonal network of leptin and male reproduction – the hypothalamus-pituitary-gonadal axis

Leptin is not merely restricted at regulating whole-energy homeostasis, it is also associated with key functions in other systems. In fact, leptin effects are pleiotropic and can be observed, for example, in the respiratory system, immune responses, cardiac function, fetal development and interestingly, in reproductive function (Lam and Lu 2007, Malli, Papaioannou et al. 2010, Garcia-Galiano, Allen et al. 2014, Chen and Yang 2015). This influence is exerted through actions in the hypothalamus and pituitary. In reproductive system, these changes induce alterations in the HPG axis. This axis has an important impact

in the control of testicular function and cells, including Leydig cells and SCs, that are crucial for a regular steroidogenesis and spermatogenesis, respectively (Johnson, Thompson et al. 2008). Furthermore, the link between leptin and reproductive function is strong, since this hormone is primarily associated with whole body energy status and reproductive function is a highly energy demanding process. However, despite early studies pointing towards an important association between energy status and reproductive capacity, it was not until leptin showed to advance the onset of puberty in mice and rescued infertility in ob/ob mice that this hormone emerged as a key element connecting these two parts (Kennedy and Mitra 1963, Chehab, Mounzih et al. 1997, Mounzih, Lu et al. 1997).

HPG axis plays an important role controlling testicular function. GnRH, a hormone released by hypothalamus, stimulates the pituitary to release LH and FSH, which control steroidogenesis and spermatogenesis, respectively. It is well established that hypothalamus, a central regulator of several functions including energy balance and reproductive function, is the primary target site of leptin due to leptin's ability to modulate the expression of several hypothalamic neuropeptides (Kwon, Kim et al. 2016). Leptin has a stimulatory effect on LH pulsatility in several species, which indicates that GnRH neurons are a downstream target of leptin (Carro, Pinilla et al. 1997, Gonzalez, Pinilla et al. 1999). However, despite LepRb being highly expressed in hypothalamus, GnRH neurons do not express it (Louis, Greenwald-Yarnell et al. 2011). LepRb is expressed in several different tissues and organs, which could indicate that the ability of leptin to regulate reproduction is not restricted at a single cell type or organ. However, re-expression of LepR in the brain of mice without LepRb restored fertility for both genders, evidencing the central role of the brain in fertility regulation through leptin action (de Luca, Kowalski et al. 2005). Since GnRH neurons do not express LepRb gene, leptin actions there seem to be mediated by intermediate neurons. Kisspeptin emerged as major candidate for this role, due to being highly recognized as excitatory stimuli upstream of GnRH in HPG axis. Interestingly, contrarily to GnRH neurons, kisspeptin neurons located in ARC express LepRb (Louis, Greenwald-Yarnell et al. 2011). Additionally, leptin administration increased hypothalamic *Kiss1* mRNA in streptozotocin-induced diabetic male rats and cell lines (Castellano, Navarro et al. 2006, Luque, Kineman et al. 2007). Depolarization of kisspeptin neurons was also observed in ARC after leptin administration and leptin-deficient ob/ob mice showed reduced *Kiss1* mRNA in ARC that is partly restored after leptin treatment (Smith, Acohido et al. 2006, Qiu, Fang et al. 2011). Altogether, leptin seems to activate GnRH neurons through activation of kisspeptin neurons in ARC, indirectly modulating the HPG axis. Furthermore, there seems to be in place a complex interplay between anorexigenic, orexigenic and kisspeptin neurons that regulates energy balance and communicates this information into the hypothalamus, which responds through GnRH production accordingly, regulating other functions such as reproductive function. However, the underlying mechanisms remain unknown.

Leptin has a clear role in puberty onset, an important event in the organism that is gated by multiple metabolic factors, that informs the reproductive system about the energy status, so that the pulse of GnRH can be triggered. Leptin and LepR deficient individuals and mice are infertile and fail to advance to puberty unless treated with exogenous leptin (Farooqi, Jebb et al. 1999). In male rats, leptin administration causes a dose-dependent stimulation of GnRH production (Parent, Lebrethon et al. 2000). Furthermore, leptin administration in normal female mice also anticipates the normal onset of reproductive function (Ahima, Dushay et al. 1997). In a pre-puberty stage in male humans, leptin values peak right before puberty (Brandão, Lombardi et al. 2003). This peak precedes the rise in free testosterone happening during puberty. Interestingly, women have higher concentrations of leptin than men, which suggests that sex differences in leptin concentration result from differences between sex hormones, such as estradiol and testosterone (Wells 2007). In fact, as mentioned before, leptin as an inhibitory effect in testosterone production and estrogens stimulate leptin release from adipocytes, which could explain the decrease in serum leptin concentrations after puberty in men, whereas in women higher concentrations of leptin are found in later stages of puberty (Shimizu, Shimomura et al. 1997, Tena-Sempere, Pinilla et al. 1999). Altogether, these findings clearly represent a crucial role for leptin in puberty onset, which means that leptin indirectly affects GnRH production.

Leptin administration stimulates the HPG axis in ob/ob mice. Characteristically infertile, following leptin administration these mice exhibit a normalization of fertility parameters (Cleary, Bergstrom et al. 2001). Furthermore, leptin administration in animals with low levels of gonadotropins due to diet restrictions, reverses this state with increases in gonadotropins levels, specially testosterone, due to an increase in GnRH production and consequently, also in LH production (Sirotkin, Chrenkova et al. 2008). In morbidly obese men, leptin resistance induces hypogonadotropic hypogonadism due to lower levels of circulating gonadotropins, which consequently induces apoptosis of testicular cells (Tapanainen, Tilly et al. 1993, Aggerholm, Thulstrup et al. 2008). Altogether, these findings indicate a role for leptin in the regulation of the HPG axis and GnRH, FSH and LH hormones.

Leptin also seems to have a direct influence in pituitary. In fact, LepR is expressed in gonadotropes both in pair tuberalis and in pair distalis (Iqbal, Pompolo et al. 2000). Leptin has shown to stimulate luteinizing hormone-releasing hormone (LHRH) in hypothalamic explants and both FSH and LH release from anterior pituitary in adult male rats *in vitro*. However, other studies have shown that leptin can only stimulate LH and not FSH release (Yu, Kimura et al. 1997). Nevertheless, leptin seems to be involved in the release of gonadotropins. Nitric oxide seems to be involved in this mechanism, as the use of a nitric oxide synthase competitive inhibitor has shown to have no effect in the basal release of LH; however, it suppressed stimulation of LH release induced by leptin. Leptin seems to act at a hypothalamic and pituitary level stimulating nitric oxide release, presumably through

receptors in both, hypothalamus and pituitary, which results in LHRH or LH release, respectively (Yu, Walczewska et al. 1997). In other studies, both gonadotropes and somatotropes have shown to locally produce leptin after a stimulus by GnRH and NPY (Akhter, Johnson et al. 2007, Akhter, Crane et al. 2011). In fact, leptin seems to signal nutritional deprivation to these cells, which lowers gonadotropins production to conserve resources explaining, in part, the lower levels of LH, promoting survival over reproduction. These findings suggest paracrine actions of leptin in the pituitary, probably through nitric oxide, however the exact mechanisms and implications on male reproduction remain undisclosed.

6. Leptin actions in reproductive function

Through the last decades, leptin has shown to have a preponderant role in reproduction. In fact, the effects of leptin in female fertility are already well established (Perez-Perez, Sanchez-Jimenez et al. 2015). However, in male reproduction there are still some doubts concerning the role of leptin as a regulator of male fertility and thus, it is still a matter of debate. Besides actions in the hypothalamus, leptin also acts in the testes. In humans, hyperleptinemia has been proposed as a negative factor in spermatogenesis and steroidogenesis (Figure 6) (Isidori, Caprio et al. 1999). Leydig cells are the major sites of androgens production in the testis. These cells are under control from pituitary LH and are crucial for the reproductive system. Higher values of leptin seem to negatively affect serum testosterone levels, through an inhibition of Leydig cells mediated by LepRb (Caprio, Isidori et al. 1999). In the testis, leptin is suggested to function as an inhibiting factor for steroidogenesis, which can explain the link between higher levels of leptin in obese men and decreased testosterone production. This effect may be due to leptin-induced dose-dependent decreased testicular expression levels of StAR, P450SCC and steroidogenic factor-1, upstream elements of steroidogenic pathway (Tena-Sempere, Manna et al. 2001). Moreover, the inhibitory effects of leptin in steroidogenesis were also observed in peripheral tissues such as the ovaries and adrenal glands, corroborating leptin inhibitory actions in this process (Kruse, Bornstein et al. 1998, Serke, Nowicki et al. 2012). In addition, there is an increased aromatization of testosterone to estradiol in peripheral fat tissues in obese men (Cohen 1999), which also contributes to the observed decreased levels of testosterone in those men. In fact, leptin can also regulate estrogen synthesis through these mechanisms in the prostate, which illustrates other mechanisms whereby leptin can regulate testosterone levels (Alves-Pereira, Colli et al. 2012).

Leptin has already several well documented effects in (in)fertility. In fact, leptin was shown to decrease the number of germ and Leydig cells, the weight and volume of testes and the diameter of seminiferous tubules in mice. Offspring of hyperleptinemic mice was also

decreased and the number of spermatogonia was increased (Yuan, Huang et al. 2014). Additionally, leptin presence in human seminal plasma together with LepR being present in spermatozoa indicates a currently unknown role for leptin modulating sperm function (Leisegang, Bouic et al. 2014). Leptin can cross the BTB, being present in seminiferous tubules and spermatozoa (Banks, McLay et al. 1999). Due to this trait, leptin from other parts of the body can influence the environment around spermatogenesis, which can be one of the mechanisms through which leptin regulates reproductive function. In obesity, spermatic function is heavily altered with reduced sperm motility, concentration and viability, which leads to a reduction in the fertility potential of these individuals (Hammoud, Wilde et al. 2008, Fernandez, Bellentani et al. 2011). Ob/ob mice are also known to present impaired spermatogenesis. Furthermore, pro-apoptotic testicular genes are also upregulated, leading to an increase of germ cell apoptosis, which could explain, in part, the decrease of fertility rates present in obesity (Bhat, Sea et al. 2006). Additionally, LepRb has been recently detected in SCs in humans, suggesting an important role of leptin in these cells (Martins, Moreira et al. 2015). SCs have a unique metabolism, exhibiting a “Warburg-like” metabolism, favoring a highly glycolytic flux to support the nutritional needs of germ cells, similarly to what happens in cancer cells. Indeed, germ cells have a complete metabolic dependence on SCs, the latter being responsible for lactate production, an essential substrate for germ cell development. Moreover, SCs are also highly susceptible to internal and external factors, such as hormones (Oliveira, Martins et al. 2015).

Recently, leptin has shown to modulate mitochondrial dynamics, biogenesis and functioning in cancer cells. Specifically, a concentration of leptin that is also found in obese patients shifted ATP production from glycolysis to mitochondria, which led to a decrease in lactate production (Blanquer-Rossello, Santandreu et al. 2015). Due to the similarities shared between SCs and cancer cells metabolism, one can hypothesize that the same mechanism can occur in SCs, but further studies are needed. In addition, identification of LepRb in SCs together with the high susceptibility of SCs to hormones, could explain the increased apoptosis in germ cells and decrease of fertility rates found in obese patients. In this case, higher concentrations of leptin can shift ATP production to mitochondria, diminishing lactate production. Lactate has been already reported as an antiapoptotic agent in male germ cells in a dose-dependent manner and high lactate concentrations are required to sustain spermatogenesis and normal testicular function (Erkkila, Aito et al. 2002). These findings could represent a novel mechanism whereby leptin regulates germ cell apoptosis through mitochondria and SCs, shifting ATP production. This would culminate in decreased sperm quality and consequently, in a decreased fertility potential found in obese individuals. Nevertheless, further studies are needed to clarify the exact role of leptin concerning male reproduction.

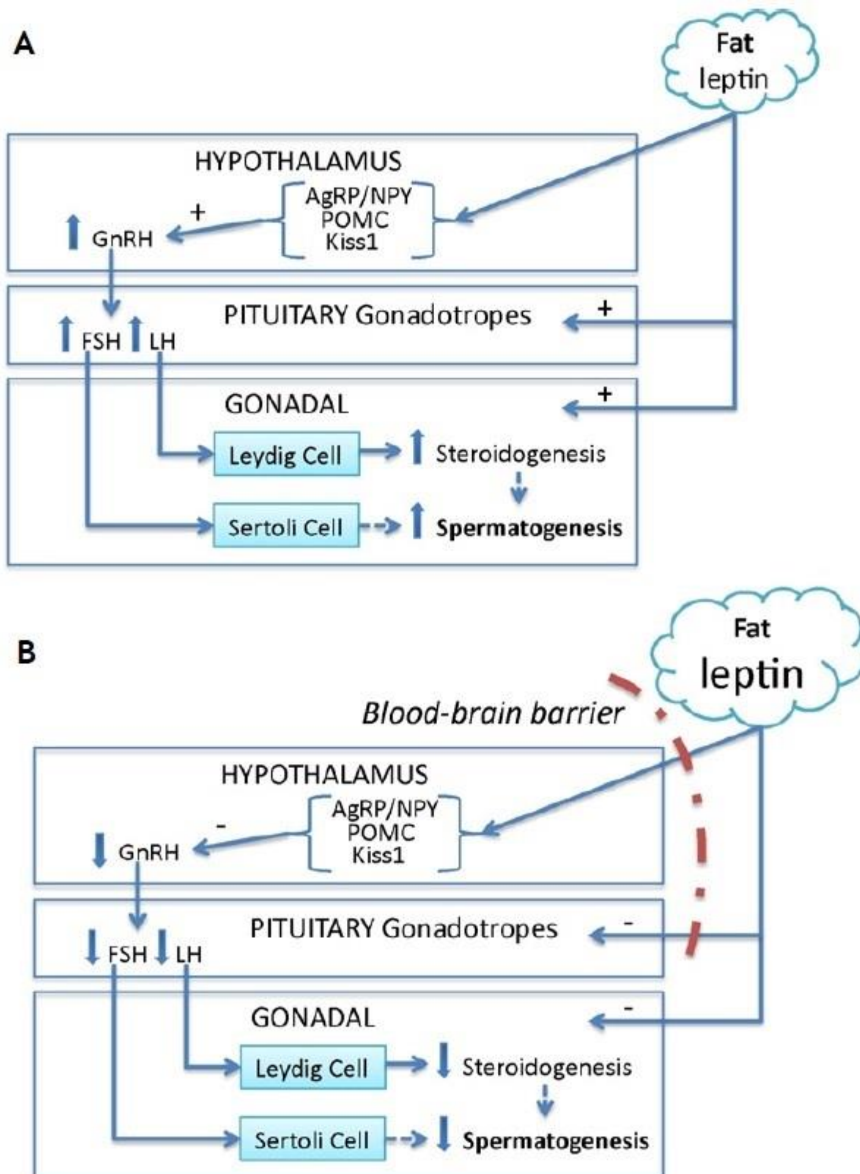

Figure 6: Leptin actions in the hypothalamic-pituitary-gonadal (HPG) axis under normal and pathological conditions. A) In an individual with a normal body mass index, leptin is produced in the adipose tissue and travels to the hypothalamus, interacting with anorexigenic, oxiregenic and kisspeptin neurons leading to an increase in GnRH production. Leptin also stimulates pituitary gonadotropes secretory activity. This causes an increase in Leydig cell steroidogenesis through stimulation by LH together with a stimulation of Sertoli cells through FSH, further stimulating spermatogenesis. B) In an obese individual, leptin inability to stimulate hypothalamic neurons and pituitary gonadotropes leads to lower levels of LH and FSH in the testis, compromising steroidogenesis and spermatogenesis. Abbreviations: GnRH – gonadotropin releasing hormone, LH – luteinizing hormone, FSH – follicle-stimulating hormone. Adapted from (Landry, Cloutier et al. 2013).

6.1 Obesity – a major cause for (in)fertility

Nowadays, metabolic diseases such as obesity and diabetes are highly recognized as severe problems, specifically in developed countries, and are considered major disruptors in the currently human reproductive health (Mitchell, Catenacci et al. 2011). These diseases are characterized by a strong hormonal dysregulation, in which the leptin-ghrelin axis controlling food intake and energy homeostasis is often dysregulated.

Obesity, a state attributed to an individual with a body mass index (BMI) of more than 30 kg/m^2, has been spreading worldwide in both, developed and developing countries, entailing severe consequences such as cardiovascular diseases, respiratory disorders, diabetes, immune deficiencies, some types of cancers and subfertility (Meldrum, Morris et al. 2017). According to the World Health Organization, in 2014 approximately more than 1.9 billion adults were overweight (BMI over 25 kg/m^2) and 38% of the adult male European population was overweight as well. Furthermore, nearly 3 million people per year die from being overweight or obese, which places obesity in the top 10 of death causes in the world. Several studies have shown a strong correlation between the increased levels of obesity during the last decades and the decline of sperm counts and consequently male fertility, which represents a major problem to public health (Paasch, Grunewald et al. 2010, Eriksson, Haring et al. 2017). A recent study showed that sperm concentration has decreased nearly 53% between 1973 and 2011 with total sperm count following a similar trend, with a decrease of 60% in men from the United States, Europe, Australia and New Zealand (Levine, Jørgensen et al. 2017). Furthermore, an inverse correlation exists between total sperm count and BMI (Paasch, Grunewald et al. 2010). Obese men have reduced sperm concentration and total sperm count, which could be responsible for fertility problems (Du Plessis, Cabler et al. 2010). Additionally, obesity is also associated with lower testosterone concentrations and a greater testosterone production decrease with age (Derby, Zilber et al. 2006). These lower concentrations are also associated with an accumulation of abdominal fat by reducing lipolysis in that area, which creates a loop, progressively exacerbating obesity (Xu, De Pergola et al. 1990, Marin and Arver 1998). Besides the well-known testosterone functions in reproductive processes, its role in avoiding andropause onset is also important. In fact, lower blood testosterone concentrations are implicated in a lower life span and the development of cardiovascular diseases in men (Bray 2004). A high-fat diet, characteristic of obesity, also leads to lower sperm motility and oxidative stress (Zhao, Zhai et al. 2014). Interestingly, leptin signaling has shown to be modulated by reactive oxygen species (ROS), which could be another link between leptin signaling and reproduction (Palomba, Silvestri et al. 2015). However, despite intense focus during the last decades of the scientific community in this topic, the mechanisms underlying metabolic balance and reproductive capacity are not yet fully understood.

6.2 Mitochondria in male reproduction – a connection with leptin

Mitochondria are pivotal organelles present in almost all eukaryotes, being responsible for multiple important functions such as oxidative phosphorylation (OXPHOS) and the citric acid cycle (also known as Krebs cycle). Frequently referred as the powerhouse of the cell, due to their role in the production of ATP needed to sustain life, mitochondria are also heavily linked with ROS production, specifically during OXPHOS (Scheibye-Knudsen, Fang et al. 2015). First named in 1898, curiously due to their identification during spermatogenesis, mitochondria are complex in form and structure. Mitochondria are constituted by more than a thousand proteins, varying between cell and tissue types, with the majority encoded by nuclear genes and a smaller number, thirteen, encoded by the maternally inherited mitochondrial DNA (Gray 2015). Due to their importance in cellular processes, they are constantly monitored, which requires bidirectional pathways mediating the crosstalk between mitochondria and the nucleus, modulating a response accordingly to developmental and environmental changes. Recent studies have switched focus from the traditional roles of mitochondria in OXPHOS and Krebs cycle and started paying attention to mitochondria functions, such as ROS production and their emerging role in cell signaling in both normal and pathological processes, steroid hormones production important in several processes, epigenetics and the central role of mitochondria in several signaling pathways (D'Aquila, Bellizzi et al. 2015, Shadel and Horvath 2015, Martin, Kennedy et al. 2016).

The role of mitochondria in male reproduction is heavily tied with sperm, with several proposed roles for mitochondria in sperm maturation, capacitation and motility. Furthermore, mitochondria also seems to be involved in providing energy for sperm survival and in sperm quality control through apoptosis (Piomboni, Focarelli et al. 2012). Spermatogenesis, a process taking place in seminiferous tubules where haploid spermatozoa are produced, is also suggested to have mitochondria involvement. In fact, mitochondria presence in male germ cells together with the presence of several testis specific mitochondrial protein isoforms corroborates their importance and involvement in testicular metabolism (Meinhardt, Wilhelm et al. 1999, Huttemann, Jaradat et al. 2003). Testis mitochondria seem to morphologically differ between cell types which could be due to mitochondrial fusion and fission processes. In fact, somatic and germ cells have distinct metabolic activities, which then translates into different mitochondrial contributions not explained due to substrate availability. For example, spermatocytes, which are closer to luminal regions of seminiferous tubules and so farther away from the oxygen source (blood vessels), prioritize OXPHOS, while spermatogonia located on the basal membrane, and so closer to the oxygen supply, prioritize glycolysis (Nakamura, Okinaga et al. 1984, Ramalho-Santos, Varum et al. 2009).

One of the key functions of mitochondria in spermatogenesis is the regulation of apoptosis, in order to ensure that a viable number of germ cells can be supported by the

existing SCs. Several studies have shown that the deletion of pro-apoptotic proteins causes an increase in germ cells and testicular tumorigenesis (Coultas, Bouillet et al. 2005, Katz, Fisher et al. 2013). Interestingly, in human sperm, mitochondrial DNA (mtDNA) copy number is present in less quantity than in oocyte (Wai, Ao et al. 2010). Lower levels of mtDNA copy number are important to a correct sperm function. In fact, men with oligozoospermia and asthenozoospermia have high levels of mtDNA in sperm (Tremellen 2008). It seems that the decrease in mtDNA copy number found in normal men is caused by the downregulation of mitochondrial biogenesis regulators during spermatogenesis (Larsson, Oldfors et al. 1997). This could indicate that the reduced number of mtDNA in sperm present in normal men is an evolutionary adaptation, which decreases sperm susceptibility to ROS damage that occurs during OXPHOS. ROS are highly reactive molecules that due to their reactive capacities can oxidize several cell constituents, such as DNA and proteins, compromising cell integrity (Ray, Huang et al. 2012). Thus, ROS levels must be kept in safe levels, otherwise an imbalance between ROS and the antioxidant defenses, such as catalase and superoxide dismutase, can occur. This condition of oxidative stress is a common characteristic of several pathologies including cancer and interestingly, insulin resistance (Sosa, Moline et al. 2013, Keane, Cruzat et al. 2015). ROS could have a detrimental effect in sperm function and overall sperm quality. In a study by George *et al.*, mice with a mutation in the inner mitochondrial membrane peptidase 2-like presented impaired processing of signal peptide sequences from mitochondrial cytochrome c1 and glycerol phosphate dehydrogenase 2, which caused oxidative stress, impaired spermatogenesis and subfertility due to excessive ROS (George, Jiao et al. 2012). Furthermore, the higher levels of mtDNA found in oligozoospermic and asthenozoospermic men could explain the defects in sperm number and function and, consequently, male infertility, present in these conditions. One of mtDNA copy number regulators is the mitochondrial transcription factor A (TFAM). This factor regulates the transcription of mtDNA and the expression of germ cell specific isoforms during spermatogenesis has been reported in mice and humans (Larsson, Oldfors et al. 1997). Additionally, its importance is highlighted in homozygous TFAM knockout mice that die early during an embryonic stage. Downregulation of TFAM in spermatids also coincides with lower mtDNA copies (Larsson, Garman et al. 1996). Altogether, TFAM seems to be involved in the regulation of mtDNA copy number during spermatogenesis; however, further studies are needed to support and prove this hypothesis. Mitochondria also seem to have an important role in sperm function. In fact, after spermiogenesis, most of mitochondria are lost; however, several mitochondria are found near the midpiece of the spermatozoa (Ho and Wey 2007). Following the principle that if they are there it is because they have a purpose, otherwise they would be lost together with most of the cytoplasm in the end of spermiogenesis, they seem to be involved in ATP production. In fact, mitochondrial inhibition impairs sperm activity and mitochondrial parameters also positively correlate with sperm quality (St John, Jokhi et al. 2005, Sousa, Amaral et al. 2011). Administration of mutant mtDNA in mice also resulted in male infertility with similar results being obtained in humans (St John, Jokhi et al.

2005, Nakada, Sato et al. 2006). However, sperm motility seems to be dependent on several energy sources, with sperm cells exhibiting a great versatility in their metabolism modulating their energy production according to the available substrates present in the female reproductive tract.

Leptin actions seems to be linked with mitochondria in several aspects. Several studies have shown that leptin actions in hypothalamus are partially mediated by ROS production. An increase in ROS activates POMC neurons, triggering leptin associated effects, whereas suppression of ROS increases NPY/AgRP neurons activity (Diano, Liu et al. 2011). ROS levels in POMC neurons seem to be partly mediated by peroxisomes, which are intracellular organelles known to be involved in the catabolism of very long chain fatty acids and ROS reduction (Bonekamp, Völkl et al. 2009). In fact, a higher number of peroxisomes was observed in POMC neurons of DIO mice when compared with lean mice. Peroxisome proliferation in hypothalamus could be explained due to the metabolic stress that ER is under in obesity, a well-known factor of leptin resistance. This could represent a novel mechanism of leptin resistance through the decreased activity of POMC neurons by peroxisomes, even in the presence of higher concentrations of leptin, leading to a diminished leptin effect. Peroxisomes proliferation is mediated, in part, by a peroxisome proliferator-activated receptor-γ (PPAR-γ). PPAR-γ is a nuclear receptor that functions as a transcription factor, regulating the expression of target genes (Green 1995). Administration of a PPAR-γ agonist in lean mice leads to a higher number of peroxisomes in POMC neurons, accompanied with a decrease in the number of ROS and an increase in food intake (Diano, Liu et al. 2011). Furthermore, administration of a PPAR-γ antagonist in DIO mice leads to the opposite with a lower number of peroxisomes in anorexigenic neurons, higher ROS levels and a reduction of daily food intake. PPAR-γ is highly expressed in ejaculated spermatozoa (Aquila, Bonofiglio et al. 2006). It is also expressed in somatic and germ cells where it seems to be involved in regulating the expression patterns of lipid metabolic genes with the ultimate goal of providing energy for spermatogenesis (Thomas, Sung et al. 2011). Furthermore, PPAR-γ has shown to have important roles in sperm capacitation, increasing the motility of capacitated spermatozoa, and sperm metabolism (Santoro, Guido et al. 2013). PPAR-γ also shares signaling pathways with leptin, as PPAR-γ promoter region is downstream of the JAK/STAT pathway, involved in leptin signaling (Davoodi-Semiromi, Hassanzadeh et al. 2012). Altogether, these findings demonstrate an important role of PPAR-γ regulating whole-body energy balance through changes in POMC neurons to leptin sensitivity mediated by ROS and in reproductive control, with important functions in sperm development.

Several studies have also shown leptin ability to modulate mitochondrial dynamics and biogenesis in several systems. Singh *et al.*, have shown that leptin administration in liver of ob/ob mice decreases basal metabolic rate, due to a reduction in mitochondrial volume density. Additionally, leptin also decreased protein levels of several substrate oxidation

system components (Singh, Wirtz et al. 2009). Furthermore, lower mRNA levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*), nuclear respiratory factor 1 (*NRF1*) and *TFAM*, together with lower mtDNA content and lower mitochondrial complexes activity, are present in cardiomyocytes from ob/ob mice (Yan, Zhang et al. 2013). A study by Li *et al*. also demonstrated that in wild-type mice, physical activity induces mitochondrial biogenesis with SIRT1-dependent PGC-1α deacetylation, which may require AMPK activation. However, in ob/ob mice, no differences occurred between pre-training and after physical activity, which could indicate that leptin is somehow required and involved in mitochondrial biogenesis. Furthermore, treatment of a myoblasts cell line with leptin resulted in increased AMPK phosphorylation and PGC-1α deacetylation (Li, Pan et al. 2011). In another study, leptin was administrated to wild-type and ob/ob mice. Before leptin administration, the liver and oxidative soleus muscle of ob/ob mice presented reduced expression of TFAM, a state reverted after leptin treatment, corroborating the hypothesis of leptin modulating mitochondrial biogenesis (Holmstrom, Tom et al. 2013). Additionally, a recent study in a cell line of breast cancer cells also demonstrated that leptin administration up-regulated genes and proteins that are involved in mitochondrial biogenesis and dynamics, such as PGC-1α and TFAM (Blanquer-Rossello, Santandreu et al. 2015). Altogether, leptin seems to be tightly involved with mitochondria at a signaling level, due to the growing importance of ROS in leptin signaling. Additionally, several findings point towards a role for leptin in the modulation of mitochondrial biogenesis, which could disrupt leptin signaling and interfere with mitochondrial well-established functions in male reproduction.

II. Aim of the Project

Metabolic diseases, such as obesity, are recognized as severe health problems and considered as major disruptors in human fertility. Male obesity in men in reproductive age has risen during the last decades, coinciding with an increase in male infertility worldwide. Furthermore, sperm quality (concentration and count) has been reported to decline at an accelerated rate during the last decades, reaching worrying levels. These factors are interconnected and have been overlooked in the last years.

Obesity disrupts whole body metabolism, particularly through actions mediated by hormones. Leptin, a hormone heavily increased in obese individuals, has proved to exert important functions in peripheral tissues. Although leptin has been on the spotlight for the last two decades, there is not much information available about its effects on male reproductive tract, particularly on SCs. Leptin has shown the ability to modulate mitochondrial dynamics and biogenesis in several systems though in different ways. Until now, the role of leptin in mitochondrial functionality and biogenesis in SCs remains unknown.

In this study, we aimed to unveil the role of leptin in mitochondrial dynamics and biogenesis in SCs, since those processes may have important implications in the nutritional support of spermatogenesis by those cells.

III. Materials and Methods

1. Chemicals

Fetal Bovine Serum (FBS) was obtained from Biochrom AG (Berlin, Germany). Insulin-transferrin-sodium selenite supplement (ITS supplement) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye were purchased from Life Technologies (Gaithersburg, MD, USA). Mammalian Protein Extraction Reagent (M-PER) and BCA Protein Assay Kit were obtained from Thermo Scientific (Whalthan, MA, USA). Dried milk was obtained from Regilait (Saint-Martin-Belle-Roche, France). Sulforhodamine B (SRB) was purchased from Biotium (Hayward, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). WesternBright™ ECL substrate was purchased from Advansta (Menlo Park, CA, USA). Leptin was obtained from Bachem (Bubendorf, Switzerland). NZY M-MuLV Reverse Transcriptase (M-MLV RT), random hexamer primers, dNTPs, NZTaq Green Master Mix, Greensafe, NZY qPCR Green Master Mix and NZYDNA Ladder VI were obtained from NZYTech (Lisboa, Portugal). Primers were obtained from STABVIDA (Oeiras, Portugal). Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12 (DMEM: Ham's F12), Ethylene Diamine Tetra Acetic acid (EDTA), Bovine Serum Albumin (BSA), trypsin–EDTA and other chemicals were all purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

2. Cell line culture

Rat Sertoli cells (SerW3 cell line) were purchased from DSMZ (Braunschweig, Germany). Briefly, rat SCs (rSCs) were cultured in 75-cm^2 flasks (VWR collection, Amadora, Portugal) at 37ºC, 5% CO_2 maintained in Sertoli culture medium (mixture of DMEM:Ham's F12 1:1, supplemented with 10% heat inactivated FBS, 50 µg/mL gentamicin, 50 U/mL penicillin, 50 mg/mL streptomycin sulfate, 0.5 mg/mL fungizone and 15 mM HEPES at pH 7.4). SerW3 is a Sertoli cell line derived from immature Wistar rSCs, immortalized by the T antigen of Simian virus (SV40), which displays morphological and functional characteristics of the native cell (Pognan, Masson et al. 1997).

Clonetics™ human Sertoli cells (MM-HSE-2305) were purchased from Lonza (Walkersville, MD, USA). hSCs were thawed following manufacturer's protocol. In brief, the vial with frozen cells was thawed at 33ºC and cells were placed in culture flask with Sertoli culture medium (mixture of DMEM:Ham's F12 1:1, supplemented with 10% heat inactivated

FBS, 50 µg/mL gentamicin, 50 U/mL penicillin, 50 mg/mL streptomycin sulfate, 0.5 mg/mL fungizone and 15 mM HEPES at pH 7.4). Cells were incubated at 33ºC in an atmosphere of 6% CO_2. Each "n" corresponds to a cell passage and all experiments were performed in triplicate.

3. Experimental design

rSCs and hSCs were allowed to grow until they reached 90–95% confluence, and then washed thoroughly. The medium was replaced by serum free media (DMEM:F12, 1:1, with ITS supplement: insulin 10 mg/L; transferrin 5.5 mg/L; sodium selenite 6.7 µg/L, pH 7.4). To evaluate the effect of leptin on rSCs and hSCs we defined a leptin-free group supplemented with ITS medium without leptin and three groups supplemented with leptin in increasing concentrations (5 ng/mL, 25 ng/mL and 50 ng/mL). The concentration of 5 ng/mL was selected agreeing with the physiological levels found in normal weight rats and lean humans (Krempler, Hell et al. 1998, Kiyici, Basaran et al. 2015). The concentration of 25 ng/mL was chosen based on levels reported in the literature for obese rats and humans (Krempler, Hell et al. 1998, Saravanan, Ponmurugan et al. 2014). We also found relevant to evaluate the effects of a concentration (50 ng/mL) that is reported in morbid obese humans. There are no reports of this concentration in rats, as such mouse model does not exist; nonetheless we also find important to study the effects of this supraphysiological concentration in rat SCs (Fathy and Morshed 2014). After a 24-hour treatment in an atmosphere of 5% CO_2 at 37ºC, cells were detached with a trypsin–EDTA solution and collected using standard methods (Oliveira, Sousa et al. 2009, Oliveira, Alves et al. 2011). Then, the total number of cells was determined using a Neubauer chamber and the cells were collected for protein, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) extraction and enzymatic assays. Viability was evaluated by the Trypan Blue Exclusion test for rSCs and hSCs.

4. Cell proliferation assay

The effect of leptin exposure on different concentrations in rSCs proliferation was evaluated by the colorimetric SRB assay (Orellana and Kasinski 2016). In brief, rSCs were seeded in a 48-well culture plate, left to grow until reaching 60-70% confluence and then treated with the different leptin concentrations in study for 24 hours. After treatment, cells were washed in phosphate buffered saline (PBS) and fixed in 1% acetic acid in methanol for 2 hours at -20°C. Afterwards, cells were stained with 0.05% (w/v) SRB dissolved in 1% of acetic acid for 1h at 37°C. Then, unbound SRB was removed through washing with 1% acetic acid solution. SRB bound to cell proteins was extracted with 10 mM Tris solution (pH 10) in a shaker for 10 min. The optical densities of the resulting media were determined at 490 nm.

SRB binds stoichiometrically to proteins under mild acidic conditions and can be extracted through basic conditions. The amount of dye extracted is proportional to cell mass, which provides an estimative of cellular proliferation.

5. Cell viability assay

The influence of leptin in rSCs metabolic activity was assessed through the MTT colorimetric assay (van Meerloo, Kaspers et al. 2011). In brief, rSCs were seeded in a 48-well culture plate, left to grow until reaching 60-70% confluence and then treated with the different leptin concentrations in study for 24 hours. After treatment, cells were washed in PBS and 50 µL MTT (5 mg/mL) dissolved in PBS was added to each well. Cells were then incubated at 37ºC for 2 hours, protected from light. Afterwards, cells were washed with dimethyl sulfoxide (DMSO) and 100 µL of each well was transferred to a 96-well culture plate. A blank was made with DMSO and the absorbance was read at 570 nm. MTT reduction to formazan, which is insoluble and has a characteristic purple color, is dependent on cellular mitochondrial dehydrogenases present in viable cells.

6. Mitochondrial membrane potential

Mitochondrial membrane potential in rSCs was measured using the JC-1 dye (Reers, Smiley et al. 1995). Briefly, after treatment with leptin, JC-1 was added to the media in a final concentration of 2 µM and the cells were incubated at 37°C for 30 min. Afterwards, the medium was replaced with medium without JC-1. Fluorescence intensities were then analyzed immediately using a Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek, Winooski, USA) pre-heated at 37ºC. The accumulation of JC1 dye in mitochondria depends on mitochondrial membrane potential. In healthy cells with high membrane potential, JC-1 forms aggregates that emit an intense red fluorescence that was detected at 550/590 nm (excitation/emission). However, in unhealthy cells, JC-1 remains in the monomeric form exhibiting green fluorescence that was detected at 485/535 nm (excitation/emission). The ratio of aggregates/monomers was calculated for each condition as a parameter of mitochondrial functionality.

7. Total protein extraction

Total protein was extracted from rSCs and hSCs using the M-PER buffer, supplemented with 1% protease inhibitor cocktail and 100 mM sodium orthovanadate. The cells were mixed

for 10 minutes at room temperature. The resulting suspension was then centrifugated at 14000.*g* for 20 minutes. Afterwards, the resulting pellet was discarded. Total protein concentration was determined using the Pierce™ BCA Protein Assay Kit according to manufacturer's instructions. Briefly, protein concentration was determined using different BSA concentrations as standards for calibration. Optical densities of samples were determined at 595 nm.

8. Western blot

Western blot was performed as previously described (Martins, Sa et al. 2016) to analyze individual protein levels of OXPHOS complexes. For that, protein samples (100 µg) were mixed with sample buffer (60 mM Tris.HCl, 10% glycerol, 2% SDS, 5% ß-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) and denatured for 15 minutes at 37ºC. Proteins were fractionated in 15% polyacrylamide gels and electrophoresis was carried out for 90 min. Afterwards, proteins were transferred from gels to previously activated polyvinylidene difluoride membranes (Merck Milipore, Darmstadt, Germany) in a Mini Trans-Blot® cell (Bio-Rad, Hemel Hempstead, UK) and then blocked for 3 hours in a 5% non-fat milk solution at room temperature. The membranes were incubated overnight at 4ºC with the primary antibodies and the immune-reactive proteins were detected separately using the antibodies listed in Table 1. Mouse ß-actin was used as a protein loading control. Membranes were reacted with WesternBright™ ECL and read with the Bio-Rad ChemiDoc XR (Bio-Rad, Hemel Hempstead, UK). Densities from each band were obtained using the Image Lab Software (Bio-Rad, Hemel Hempstead, UK) using standard methods. The band density obtained was divided by the respective ß-actin band density and then normalized with the control group value.

Table 1: List of the primary and secondary antibodies used in this study.

ANTIBODY	HOST SPECIE	MOLECULAR WEIGHT (KDA)	DILUTION	VENDOR	CATALOG #
OXPHOS	Mouse	20, 30, 40, 48 and 53	1:1000	Abcam, United Kingdom	ab110413
Β-ACTIN	Mouse	42	1:5000	Thermo Fisher, USA	MA5-15739
MOUSE	Goat	-	1:5000	Sigma-Aldrich, Germany	A3562

9. RNA and DNA extraction

Extraction of total RNA (tRNA) from rSCs and hSCs was performed using the GRS Total RNA Kit (GRiSP, Porto, Portugal), as indicated by the manufacturer. DNA from rSCs and hSCs was extracted using the E.Z.N.A. Tissue DNA Kit (Omega bio-tek, Norcross, GA, USA), also

following manufacturer's instructions. In both cases, RNA and DNA from rSCs and hSCs was isolated after detaching cells from the culture flasks using a trypsin-EDTA solution. To eliminate residual trypsin, detached cells were washed with 3 mL of PBS, by centrifugation at 3000.*g* for 5 minutes. RNA and DNA concentration and absorbance ratios (A260/A280) used to measure sample purity were determined using Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

10. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

tRNA obtained for each sample was reversely transcribed in a mixture containing 2.5 µL of random hexamer primers (50 ng/µL), 1 µL of dNTPs (10 mM), 1 µg of tRNA and sterile H_2O up to a volume of 17 µL. The mixture was then incubated during 5 minutes at 65ºC. Then, 1 µL of M-MLV RT and 2 µL of Reaction Buffer were added and incubated sequentially at 25ºC for 10 minutes, 37ºC for 50 minutes and 70ºC for 15 minutes. The resulting complementary DNA (cDNA) was used with exon-exon spanning primer sets designed to amplify the housekeeping gene 18S ribosomal RNA (18S) to evaluate sample integrity (Table 2). Each polymerase chain reaction (PCR) contained 1 µL of cDNA in 12.5 µL of final volume of a mixture containing 6.50 µL of NZYTaq Green Master Mix, 0.1 µL (50 µM) of each primer and sterile H_2O. Adipose tissue was used as positive control and cDNA-free sample was used as a negative control. At the end of the experiments, samples were run in 1% agarose gel electrophoresis with 2 µL of Greensafe in 200 mL, during 30 minutes at 120 V, to confirm sample integrity. The agarose gel was visualized in Bio-Rad GelDoc XR (Bio-Rad, Hemel Hempstead, UK) using the software Quantity One (Bio-Rad, Hemel Hempstead, UK). The size of the expected products was compared to a DNA ladder VI.

11. Quantitative PCR (qPCR)

mRNA expression levels of *PGC-1a*, *NRF1*, *TFAM* and Sirtuin 1 (*SIRT1*) were evaluated in the different experimental groups in rSCs and hSCs by qPCR. Specific primers were designed for target amplification and housekeeping transcripts (Table 2). qPCR was carried out in an CFX 96 qPCR setup (Bio-Rad, Hercules, CA, USA). Amplification efficiency was determined for all primer sets using serial dilutions of cDNA (1:3, 1:15 and 1:75). qPCR conditions and reagents concentrations were previously optimized and amplicons specificity was determined by melting curves. qPCR amplifications used 1 µL of diluted 1:15 cDNA in a 20 µL reaction containing 10 µL NZY qPCR Green Master Mix, 0.4 µL (10 mM) of forward and reverse primers for each gene and sterile H_2O. Amplification conditions comprised an initial

denaturation step of 5 minutes at 95ºC, followed by a specific number of runs for each set of primers of a 3 steps cycle: (1) a denaturation step of 30 s at 95ºC, (2) an annealing step of 30 s with a specific temperature for each set of primers and (3) an extension step of 1 minute at 72ºC. Target genes, sequences and annealing temperatures are described in Table 2. $ß_2$-microglobulin (ß2M) transcript levels were used to normalize the mRNA expression levels of target genes. Fold variation of the expression levels was calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$ (Pfaffl 2001).

12. Mitochondrial DNA copy number determination

mtDNA copy number in rSCs and hSCs was assessed through qPCR analysis, as described before. Briefly, the reaction mixture consisted in 10 µL of NZY qPCR Green Master Mix, 0.4 µL (10 mM) of forward and reverse primers, 20 ng of mtDNA and sterile H_2O. qPCR was carried out in an CFX 96 qPCR setup (Bio-Rad, Hercules, CA, USA). Nuclear encoded $ß_2$-microglobulin (*$ß2M_{nc}$*) gene transcript levels were used to normalize the mtDNA expression levels of mitochondrially encoded NADH dehydrogenase 1 (*ND1*), following the mathematical model proposed by Pfaffl (Table 2) (Pfaffl 2001).

Table 2: Genes, oligonucleotide sequence and respective conditions for PCR amplification.

Gene	Sequence (5'- 3')	AT (ºC)	Amplicon size (bp)	Species of origin
PGC-1α AN: NM_031347.1	Forward: TGGAGTGACATAGAGTGTGCTG Reverse: CTGATCCTGTGGGTGTGGTT	56	491	rat
TFAM AN: NM_031326.1	Forward: GCTTTCGTGGCTGTGTGTAG Reverse: TCTTAGCACGCCCCACATTC	58	115	rat
NRF1 AN: NM_001100708.1	Forward: TACTCCACAGGTCGGGGAAA Reverse: CATACAGAAGGTCCTCCCGC	62	199	rat
SIRT1 AN: XM_008772947.1	Forward: ACACACAAAATCCAGCAACTC Reverse: GATGCTGTTGCAAAGGAACCA	60	253	rat
ß2M AN: NM_012512.1	Forward: CCGTGATCTTTCTGGTGCTTGTC Reverse: CTATCTGAGGTGGGTGGAACTGAG	58	150	rat
ND1 AN: KM577634.1	Forward: GAGCCCTACGAGCCGTTGCC Reverse: GCGAATGGTCCTGCGGCGTA	58	271	rat
$ß2M_{nc}$ AN: NM_012512.2	Forward: GCGTGGGAGGAGCATCAGGG Reverse: CTCATCACCACCCCGGGGACT	58	264	rat
PGC-1α AN: NM_013261.3	Forward: TGGTTGCCTGCATGAGTGT Reverse: CACCACTTGAGTCCACCCAG	56	253	human

NRF1 **AN: NM_005011.4**	Forward: CAGCCGCTCTGAGAACTTCA Reverse: GGCCGTTTCCGTTTCTTTCC	58	300	human
SIRT1 **AN: AF083106.2**	Forward: ACAGGTTGCGGGAATCCAAA Reverse: GTTCATCAGCTGGGCACCTA	60	155	human
B2M **AN: NM_004048.2**	Forward: ATGAGTATGCCTGCCGTGTG Reverse: CAAACCTCCATGATGCTGCTTAC	60	93	human
ND1 **AN: NC_012920.1**	Forward: CGATTCCGCTACGACCAACT Reverse: AGGTTTGAGGGGGAATGCTG	58	121	human
$B2M_{nc}$ **AN: NG_012920.1**	Forward: GAGGCTATCCAGCGTGAGTC Reverse: GACGCTTATCGACGCCCTAA	58	306	human
18S **AN: NR_046237.1**	Forward: AAGACGAACCAGAGCGAAAG Reverse: GGCGGGTCATGGGAATAA	56	149	rat/ human

Abbreviations: *PGC-1a* (peroxisome proliferator-activated receptor γ coactivator 1α); *TFAM* (transcription factor A, mitochondrial); *NRF1* (nuclear respiratory factor 1); *SIRT1* (Sirtuin 1); *B2M* (Beta-2-Microglobulin); *ND1* (mitochondrially encoded NADH dehydrogenase 1); $B2M_{nc}$ (nuclear encoded beta-2-microglobulin); *18S* (18S ribosomal RNA); AT (annealing temperature); AN (Genbank Accession Number).

13. Statistical analysis

Statistical significance among the experimental groups was assessed by t-student tests. All experimental data are shown as mean ± SEM (n=6 for each condition). Statistical analysis was performed using GraphPad Prism 6 (GraphPad software, San Diego, CA, USA). Outliers were removed using ROUT method with a q value of 1%. Results were considered significant when $p < 0.05$.

IV. Results

1. Leptin alters rat Sertoli cells metabolic activity but has no effect in their proliferation

Initially, we evaluated leptin effects in rSCs metabolic activity through MTT assay. Our results demonstrated that leptin in the different concentrations alters the metabolic activity of rSCs. In fact, a decrease in rSCs metabolic activity is observed after the exposure to 5 ng/mL of leptin (0.73 ± 0.03 - fold variation to control), 25 ng/mL of leptin (0.74 ± 0.01 - fold variation to control) and 50 ng/mL (0.68 ± 0.04 - fold variation to control) relative to the group that was not exposed to leptin (Figure 7A). Following these results, we evaluated the effect of leptin exposure in different concentrations in rSCs proliferation by SRB assay. Non-statistically different values were obtained in the group exposed to 5 ng/mL (0.88 ± 0.05 - fold variation to control), 25 ng/mL (0.96 ± 0.04 - fold variation to control) or 50 ng/mL (0.88 ± 0.03 - fold variation to control) of leptin (Figure 7B).

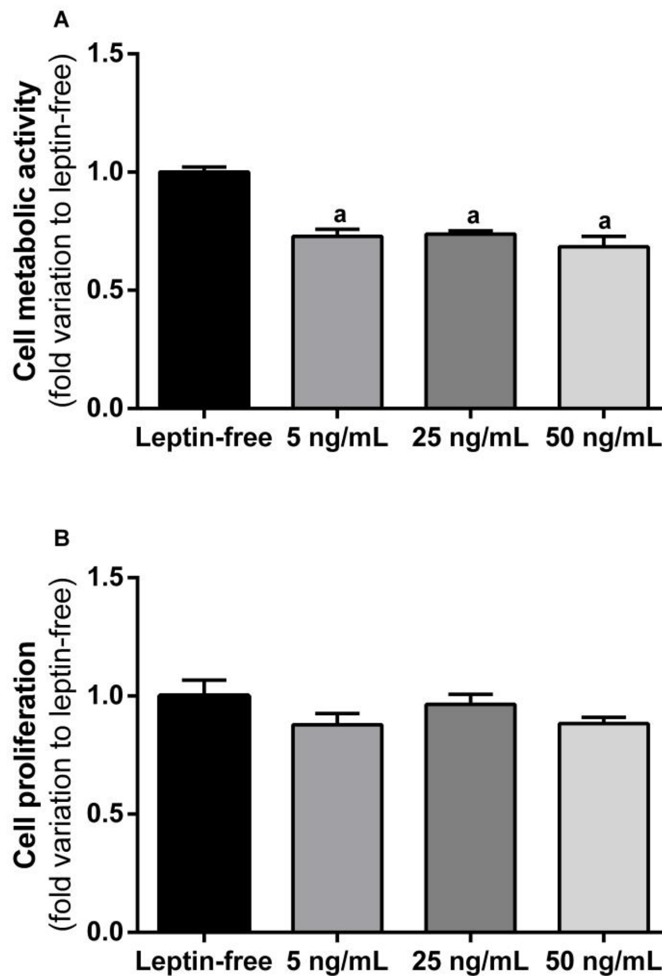

Figure 7: Effect of leptin in rat Sertoli cells metabolic activity and proliferation. The figure shows pooled data of independent experiments, indicating metabolic activity (panel A) and proliferation (panel B) of rat Sertoli cells cultured in the absence or presence (5, 25 and 50 ng/mL) of leptin. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to leptin-free group.

2. Exposure of rat Sertoli cells to a supraphysiological concentration of leptin decreases mitochondrial membrane potential

In the last years, leptin has been in the spotlight but only recently it was shown that leptin modulates mitochondrial function. Electron transport chain, located in the mitochondria, is responsible for the formation of a proton gradient that is ultimately responsible for ATP production. Indeed, it is comprised by four interconnected multi-subunit complexes (complexes I-IV) that will generate an electrochemical proton gradient across the inner mitochondrial membrane, resulting in ATP synthesis by the ATP synthase, which is usually referred as mitochondrial complex V. This electrochemical proton gradient can be measured as mitochondrial membrane potential, a key indicator of cell health and metabolic

status. Our results did not show significant differences in protein levels of mitochondrial complexes I-V in rSCs exposed to leptin in any of the tested concentrations (Figure 8A) (Table 3). However, JC-1 ratio decreased in rSCs cells exposed to the highest leptin concentration (50 ng/mL) to 0.78 ± 0.03 – fold variation to control when compared with the leptin-free group and with both, rSCs exposed to 5 ng/mL and 25 ng/mL of leptin (1.03 ± 0.06 and 1.04 ± 0.05, respectively) (Figure 8B).

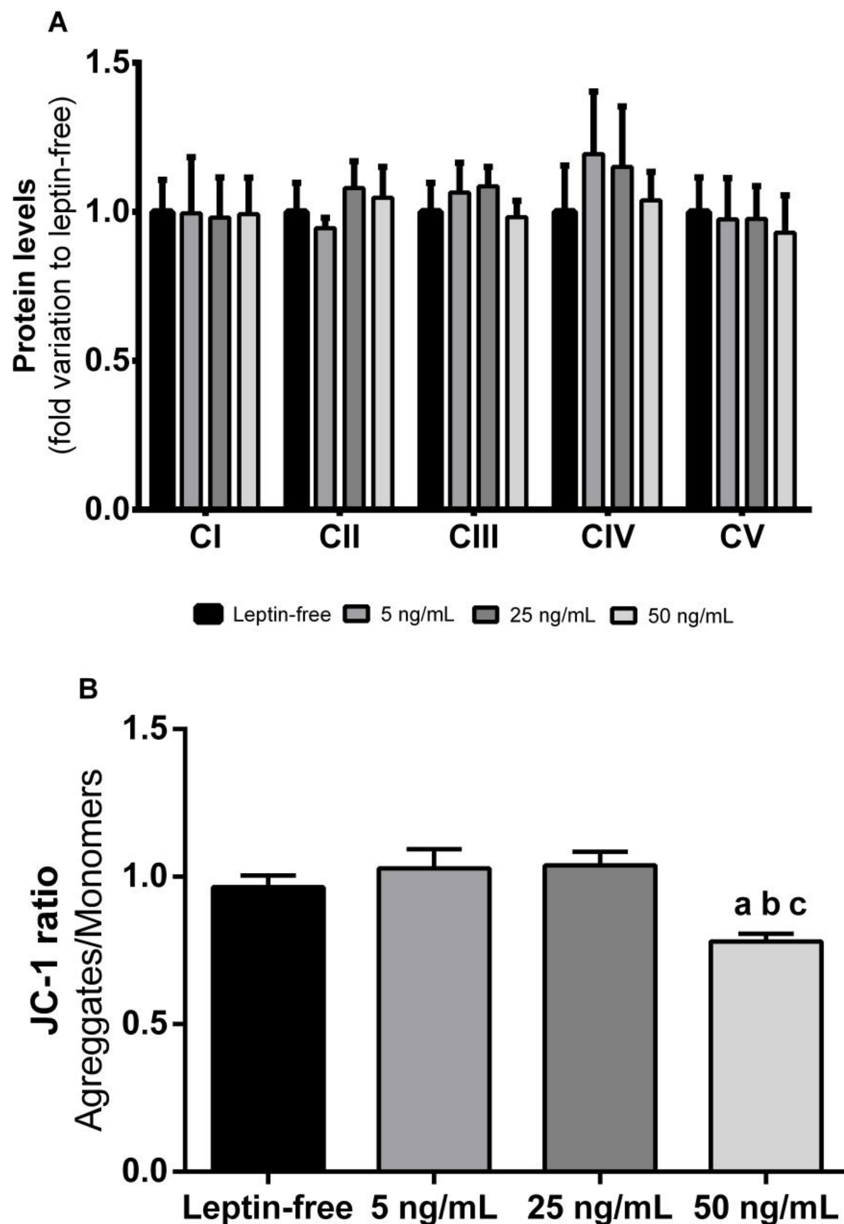

Figure 8: Effect of leptin in rat Sertoli cells mitochondria. The figure shows pooled data of independent experiments, indicating OXPHOS protein levels (panel A) and JC-1 ratio (panel B) of rat Sertoli cells cultured in the absence or presence (5, 25 and 50 ng/mL) of leptin. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to leptin-free group; b – relative to 5 ng/mL group; c- relative to 25 ng/mL group.

Table 3: Protein expression levels of mitochondrial complexes in rat Sertoli cells non-exposed or exposed to 5, 25 and 50 ng/mL of leptin.

Mitochondrial complexes	Leptin-free	5 ng/mL of leptin	25 ng/mL of leptin	50 ng/mL of leptin
CI	1.00 ± 0.10	0.99 ± 0.19	0.98 ± 0.14	0.99 ± 0.12
CII	1.00 ± 0.09	0.94 ± 0.03	1.08 ± 0.09	1.05 ± 0.10
CIII	1.00 ± 0.10	1.07 ± 0.10	1.09 ± 0.07	0.98 ± 0.05
CIV	1.00 ± 0.16	1.20 ± 0.20	1.15 ± 0.20	1.04 ± 0.10
CV	1.00 ± 0.12	0.97 ± 0.14	0.98 ± 0.11	0.93 ± 0.13

3. Leptin does not alter the expression levels of genes involved in mitochondrial biogenesis and copy number in rat Sertoli cells

In addition to modulating mitochondrial function, leptin has also shown the ability to modulate mitochondrial biogenesis. Following our previous results, we studied whether leptin could modulate mitochondrial biogenesis. For that, we analyzed the levels of four critical genes involved in such process, *PGC-1a*, *TFAM*, *NRF1* and *SIRT1*. Our results showed no statistical significant differences between the groups of rSCs exposed to increasing concentrations of leptin and the non-exposed group (Figure 9A-D). Furthermore, the analysis of the mitochondrial copy number by qPCR also revealed no statistical significant differences between the different groups of exposed and non-exposed rSCs to leptin (Figure 9E).

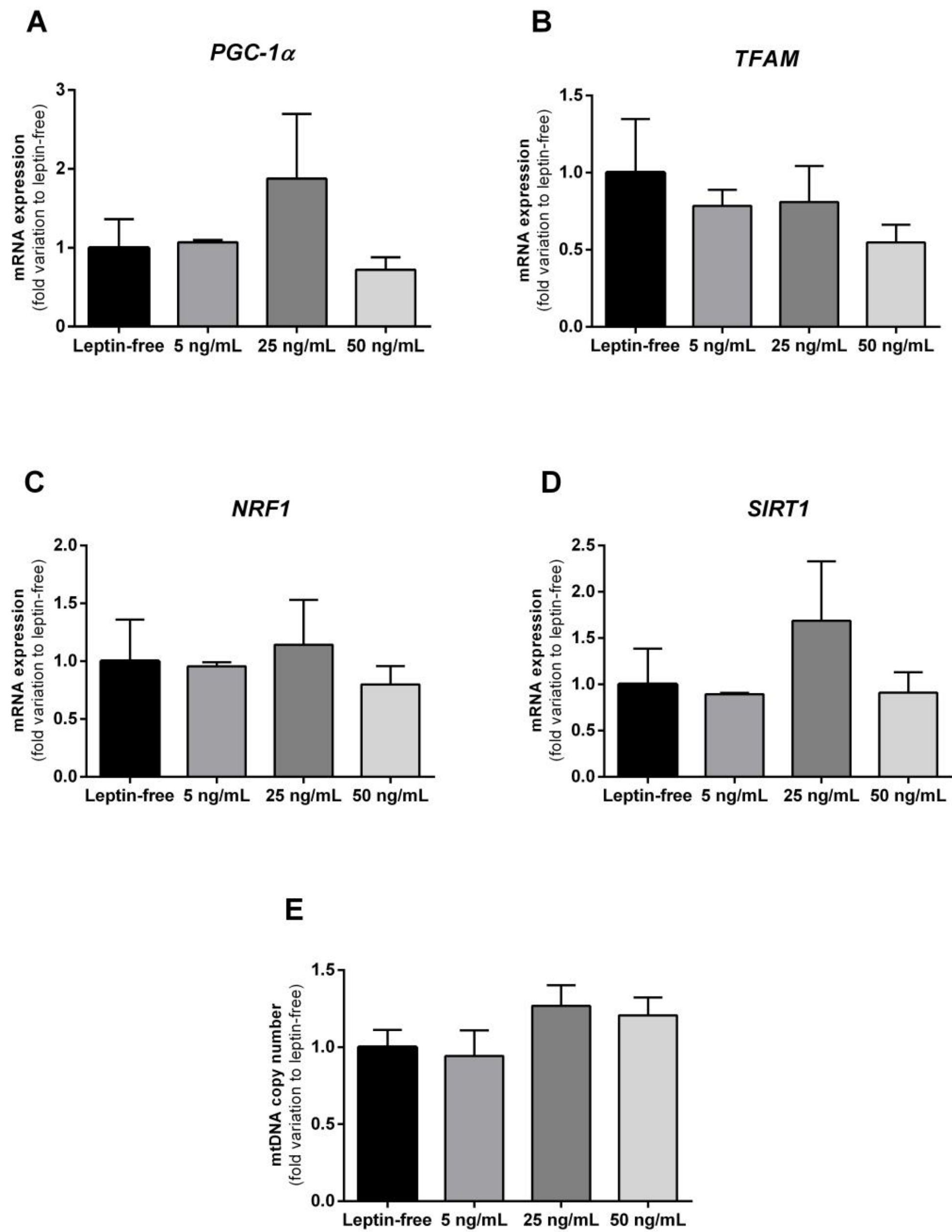

Figure 9: Effect of leptin in mRNA levels of peroxisome proliferator-activated receptor γ coactivator 1α (*PGC-1α*), transcription factor A, mitochondrial (*TFAM*), nuclear respiratory factor 1 (*NRF1*), Sirtuin 1 (*SIRT1*) and mitochondrial copy number of rat Sertoli cells. The figure shows pooled data of independent experiments, indicating mRNA levels of *PGC-1α* (panel A), *TFAM* (panel B), *NRF1* (panel C), *SIRT1* (panel D) and mitochondrial copy number (panel E) of rat Sertoli cells cultured in the absence or presence (5, 25 and 50 ng/mL) of leptin. Results are expressed as mean ± SEM (n=6 for each condition).

4. Exposure of human Sertoli cells to a supraphysiological concentration of leptin increases *SIRT1* mRNA levels

Recently, our group has identified the expression of the leptin receptor in hSCs. Furthermore, our group also reported leptin's ability to modulate the glycolytic profile of these cells. Following our initial results attained in rSCs, which did not show significant changes in mitochondrial biogenesis, we took advantage of previously published results by our team that showed that in hSCs, leptin was able to change their metabolic activity and suggested a possible action in mitochondria. Following this, we tested if leptin could modulate the mRNA levels of the previous reported genes involved in mitochondrial biogenesis in hSCs. Our results showed that mRNA levels of *SIRT1* are decreased in hSCs treated with 50 ng/mL of leptin (1.50 ± 0.09 - fold variation to control) when compared with the non-exposed group of cells and compared with both, hSCs exposed to 5 ng/mL and 25 ng/mL of leptin (0.92 ± 0.18 and 0.88 ± 0.10, respectively) (Figure 10A). However, when analyzing the mRNA levels of *PGC-1α* and *NRF1*, no statistical significant differences were observed between the different groups (Figure 10B,C). Furthermore, no statistical significant differences were observed between the different groups regarding the number of mitochondrial copies (Figure 10D).

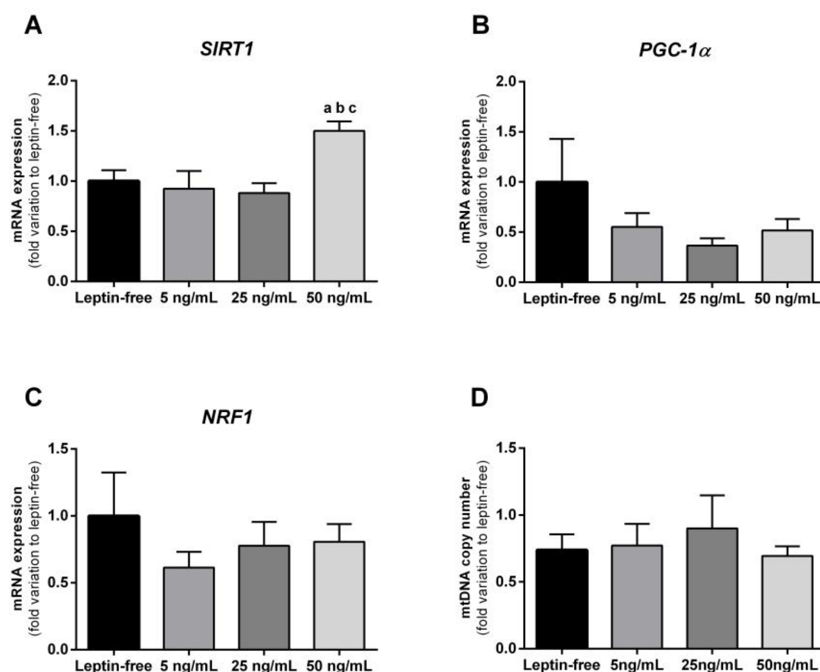

Figure 10: Effect of leptin in mRNA levels of peroxisome proliferator-activated receptor γ coactivator 1α (*PGC-1α*), nuclear respiratory factor 1 (*NRF1*), Sirtuin 1 (*SIRT1*) and mitochondrial copy number of human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating mRNA levels of *SIRT1* (panel A), *PGC-1α* (panel B), *NRF1* (panel C) and mitochondrial copy number (panel D) of hSCs cells cultured in the absence or presence (5, 25 and 50 ng/mL) of leptin. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to leptin-free group; b – relative to 5 ng/mL group; c- relative to 25 ng/mL group.

5. Exposure of human Sertoli cells to leptin modulates mitochondrial complex II expression

In order to disclose whether leptin modulates mitochondrial dynamics in hSCs, we analyzed the protein levels of mitochondrial complexes in hSCs from the different groups. Our results showed that the protein levels of complex II were decreased in hSCs exposed to 5 ng/mL of leptin (0.63 ± 0.06 – fold variation to control) and in hSCs exposed to 50 ng/mL of leptin (0.57 ± 0.08 – fold variation to control) relative to hSCs not exposed to this hormone (Figure 11). The remaining comparisons between the protein levels of complexes I-V and the different experimental groups are presented in table 4.

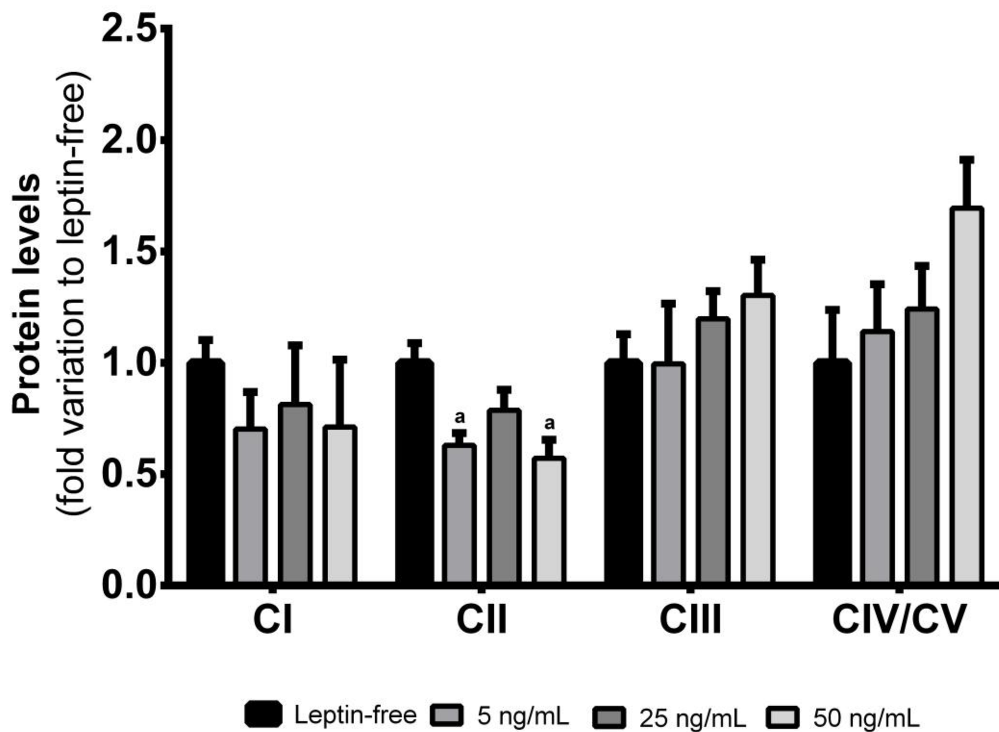

Figure 11: Effect of leptin in OXPHOS protein levels of human Sertoli cells (hSCs). The figure shows pooled data of independent experiments, indicating OXPHOS protein levels of hSCs cells cultured in the absence or presence (5, 25 and 50 ng/mL) of leptin. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results (P < 0.05) are indicated as: a – relative to leptin-free group.

Table 4: Protein expression levels of mitochondrial complexes in human Sertoli cells non-exposed (leptin-free) or exposed to different leptin concentrations (5, 25 and 50 ng/mL).

Mitochondrial complexes	Leptin-free	5 ng/mL of leptin	25 ng/mL of leptin	50 ng/mL of leptin
CI	1.00 ± 0.10	0.70 ± 0.17	0.81 ± 0.27	0.71 ± 0.30
CII	1.00 ± 0.09	0.63 ± 0.06	0.79 ± 0.09	0.57 ± 0.08
CIII	1.00 ± 0.13	0.99 ± 0.27	1.20 ± 0.12	1.30 ± 0.16
CIV/CV	1.00 ± 0.24	1.14 ± 0.21	1.24 ± 0.19	1.69 ± 0.22

V. Discussion

In the last years, metabolic diseases such as obesity are on the rise across the world, reaching worrying levels (Mitchell, Catenacci et al. 2011). These diseases are characterized by a strong hormonal dysregulation, specifically in the leptin-ghrelin axis, which regulates body energy homeostasis. Obesity is characterized by excessive or abnormal adiposity and chronic inflammation and is closely related with other major public health problems, such as type 2 diabetes mellitus, cardiovascular diseases, musculoskeletal disorders and cancer (Garg, Maurer et al. 2014, Allott and Hursting 2015). However, due to the complexity of this chronic disease, several factors have been appointed as causes for obesity development and its associated metabolic disorders, such as mitochondrial and adipose tissue dysfunction or genetic factors (Bouchard, Tremblay et al. 1990, Hajer, van Haeften et al. 2008). Current lifestyle of modern societies is also a major cause of the vertiginous increase of obesity around the world. In 2014, more than 1.9 billion adults were overweight, with nearly half of the adult male European population included in this group. Unfortunately, this number is expected to continue to dramatically increase during the next decades. Interestingly, infertility and decreased sperm quality are also following this trend. Sperm concentration and sperm count in developed countries has been on a downhill spiral approaching the minimum levels established that are required for men to be considered fertile (Levine, Jørgensen et al. 2017). Furthermore, an inverse correlation exists between BMI and total sperm count (Paasch, Grunewald et al. 2010) illustrating the correlation between these two factors. Leptin, an adipocyte-derived hormone, is heavily increased in overweight and obese individuals. Besides the well-described functions at hypothalamic level, leptin has emerged as a central player with multiple important actions already reported in peripheral tissues (Bado, Levasseur et al. 1998, Wolsk, Mygind et al. 2012). Despite several authors reporting the involvement of leptin in the metabolic control of male reproduction through its action in testicular cells, its specific role is still undisclosed (Roa and Tena-Sempere 2014). Several progresses have been made in this field and recently our group has identified the leptin receptor in SCs (Martins, Moreira et al. 2015). SCs are the testicular cells responsible for the maintenance of spermatogenesis. In fact, the metabolites resulting from their Warburg-like metabolism are fundamental for spermatogenesis. Lactate produced by these cells is the primary substrate for energy production by germ cells (Oliveira, Martins et al. 2015) and the existing concentration of this metabolite is directly associated with the number of spermatozoa produced. Thus, the metabolic cooperation that is established between SCs and germ cells is essential and, when disrupted, leads to infertility or subfertility. Leptin has shown the ability to modulate mitochondrial dynamics, biogenesis and functioning in several cellular systems, including cancer cells (Blanquer-Rossello, Santandreu et al. 2015). Notably, cancer cells metabolism is very similar to SCs, where these cells sustain a very high glycolytic flux, even in the presence of oxygen. Hence, we hypothesized that leptin might function as a metabolic regulator of

mitochondrial dynamics and biogenesis in SCs, with possible implications for the nutritional support of spermatogenesis and may unveil some molecular mechanisms responsible for the obesity-related male subfertility and/or infertility.

Previous studies have shown that leptin stimulates cell proliferation in several cellular systems, including cancer cells (Grossmann and Cleary 2012, Dubois, Jarde et al. 2014, Fazolini, Cruz et al. 2015), with leptin being regarded as a growth factor in some cancer types, such as breast cancer (Hu, Juneja et al. 2002). SCs have a characteristic proliferation pattern and their number in the adulthood determines the number of germ cells supported and consequently, male fertility potential. Notably, in all species, SCs cease to divide at adulthood (Sharpe, McKinnell et al. 2003) or present a very low proliferative rate, though this last suggestion is still a matter of intense debate (Sridharan, Simon et al. 2007, Tarulli, Stanton et al. 2012). We studied rSCs proliferation in the absence and presence of leptin at different concentrations and we detected no differences in rSCs proliferation between the different groups. These results are in line with a previous work by our group using hSCs where leptin, used in the same concentrations as in this work, also had no effect in SCs proliferation (Martins, Moreira et al. 2015). Leptin ability to stimulate cell proliferation in studies using cancer cells could be explained due to the metabolic reprogramming that cancer cells undergo (Ward and Thompson 2012) and the abundance of the necessary machinery to promote proliferation. In addition, in most of those studies, the time of exposure to leptin is higher than the one used in our work (24 hours), which may have implications in the regulation of proliferation exerted by leptin (Liu, Wang et al. 2013). Nevertheless, leptin has shown no effect on rSCs proliferation.

SCs have a characteristic metabolic activity that is crucial to sustain germ cell pool. Our results illustrate that leptin in the different concentrations used in this work decrease rSCs metabolic activity when compared with the non-treated group. These results are concomitant with previous studies in human pancreatic cancer cell lines and in human pituitary cancer cell lines, where leptin also decreased the metabolic activity of those cells (Somasundar, Yu et al. 2003, Liu, Zhong et al. 2009). Interestingly, in most studies, leptin acted in a dose-dependent manner with increasing leptin concentrations further decreasing the cell's metabolic activity. Nevertheless, our study showed no differences between the group treated with the lower leptin concentration (5 ng/mL) and the groups treated with higher leptin concentrations (25 and 50 ng/mL). Several studies in distinct cancer cell lines also demonstrated that leptin has different effects in cell metabolic activity, depending upon organ of derivation (Somasundar, Yu et al. 2003), which illustrates that leptin action in metabolic activity may be more dependent of the organ than the concentration used.

Recent studies have pointed towards an important role for leptin in modulation of mitochondrial function and dynamics. A study in liver from ob/ob mice has shown that leptin

administration in a concentration of 400 ng/µL decreases the protein levels of mitochondrial complex I and of two subunits of complex IV, which was accompanied with a reduction in mitochondrial volume density (Singh, Wirtz et al. 2009). Cardiomyocytes from ob/ob mice also present lower mitochondrial complexes activity illustrating that leptin is needed for a correct mitochondrial function (Yan, Zhang et al. 2013). In our study, exposure of rSCs to different concentrations of leptin did not altered mitochondrial complexes expression. These results are in line with a study in a human breast cancer cell line, where administration of 50 ng/mL of leptin did not produce alterations in mitochondrial complexes levels (Blanquer-Rossello, Santandreu et al. 2015). However, when analyzing the mitochondrial membrane potential, we detected a decrease after exposure of rSCs to 50 ng/mL of leptin. Leptin ability to modulate rSCs metabolism through mitochondria is associated with the decrease detected in the metabolic activity of cells exposed to 50 ng/mL, as measured by MTT assay. Although the major part of MTT cellular reduction occurs outside mitochondria, a small part still occurs inside, which could explain the decrease in rSCs metabolic activity after exposure to this concentration (Berridge and Tan 1993). These findings suggest that leptin modulates mitochondrial dynamics through changes in mitochondrial membrane potential that are reflected in the overall cell metabolic activity status for the highest concentration in study. It appears that the mechanisms by which leptin modulates rSCs are different, according with the leptin concentration. The highest concentration of leptin used in this work is present in morbidly obese humans (Fathy and Morshed 2014). In obesity, mitochondrial dysfunction is a common trait. The nutrient excess that occurs in obesity leads to mitochondrial dysfunction, which has consequences in glucose and lipid metabolism (Bournat and Brown 2010). Obesity-induced mitochondrial dysfunction seems to be caused by a series of factors, including excessive ROS production and dysregulation in the levels of genes involved in mitochondrial biogenesis (Furukawa, Fujita et al. 2004, Semple, Crowley et al. 2004). Herein, we observed a decrease in mitochondrial membrane potential after exposure of rSCs to 50 ng/mL of leptin, which reflects mitochondrial dysfunction caused by leptin levels found in morbidly obese individuals. This could represent a novel mechanism through which leptin contributes to obesity-induced subfertility or infertility in males. Nevertheless, further studies are needed to consolidate these findings.

To date, there is little information linking leptin and SCs metabolism though it is known that leptin is a pivotal modulator of metabolism in several cells and tissues. Previous studies in a breast cancer mice model showed that the absence of peripheral leptin receptors turned the metabolic phenotype less reliant on glycolysis with an increase capacity for ß-oxidation (Park, Kusminski et al. 2010). Furthermore, several studies have shown that leptin stimulates fatty acid oxidation, ROS production and glucose uptake in endothelial cells and muscle (Yamagishi, Edelstein et al. 2001, Minokoshi, Kim et al. 2002). Similar effects were observed in adipocytes, where leptin also increased the expression of genes involved in mitochondrial biogenesis, such as *PGC-1a*, suggesting an involvement of leptin with mitochondrial function

(Luo, Yu et al. 2008). Lower levels of several genes involved in mitochondrial biogenesis, such as *PGC-1a*, *NRF1* and *TFAM*, accompanied with lower number of mtDNA copies were also observed in cardiomyocytes from ob/ob mice (Yan, Zhang et al. 2013). To determine if leptin modulates mitochondria biogenesis in rSCs, we measured the expression of key markers of mitochondrial biogenesis and function. *PGC-1a* expression, the master regulator of mitochondrial biogenesis (Rohas, St-Pierre et al. 2007), was not affected in rSCs exposed to leptin in the different concentrations. Following this, we evaluated the mRNA expression of *NRF1*, since one of the PGC-1α functions is to promote its expression. NRF1 functions as a transcription factor involved in the expression of mitochondrial genome codified genes (Jornayvaz and Shulman 2010). However, we did not find statistical significant differences in mRNA levels of *NRF1* in rSCs of the different groups exposed to leptin. Lastly, we determined the mRNA levels of two genes also involved in mitochondrial biogenesis, *TFAM* and *SIRT1*. TFAM also functions as a transcription factor regulating the expression of nuclear genes required for mtDNA transcription and replication (Jornayvaz and Shulman 2010). On the other hand, SIRT1 is involved in PGC-1α deacetylation with implications in metabolic control and mitochondrial biogenesis (Tang 2016). Our results show that *TFAM* and *SIRT1* mRNA levels were also not altered after exposure to the different leptin concentrations used in this work. Interestingly, previous studies were these markers were upregulated after exposure to leptin were associated with cell proliferation (Blanquer-Rossello, Santandreu et al. 2015). However, in our study, leptin had no effect in rSCs proliferation, which was associated with no statistical significant differences between exposed and non-exposed cells in the mitochondrial biogenesis markers evaluated. The number of mitochondrial copies was also unchanged in rSCs after exposure to the different concentrations of leptin used in this work. These puzzling findings suggest that leptin does not modulate mitochondrial number or biogenesis but has an effect in mitochondrial function, particularly after exposure to 50 ng/mL. These results were at this point somewhat intriguing, taking in consideration a recent work by our group, showing that leptin modulates the glycolytic profile and acetate production, an intermediate that is also related with mitochondrial functioning, in hSCs. Thus, we evaluated if leptin could modulate mitochondrial biogenesis in these same cells (Martins, Moreira et al. 2015) to compare leptin effects between rat and human SCs.

In hSCs, our results showed that mRNA levels of *PGC-1a* and *NRF1* were not altered after exposure to leptin in the concentrations used in this study. However, in the group treated with 50 ng/mL of leptin, mRNA levels of *SIRT1* were increased relatively to the other groups. In addition to its involvement in mitochondrial biogenesis, SIRT1 function is heavily tied with the control of cellular metabolism, by functioning as a metabolic sensor. Notably, SIRT1 is involved in lipid and glucose metabolism (Li 2013, Ye, Li et al. 2017). SCs take glucose from circulation and produce metabolites essential to germ cells development. A previous study by our group has shown that leptin modulates protein levels of glucose transports, particularly GLUT2, which may be linked with obesity-related male subfertility/infertility since lower

protein levels of GLUT2 were found in a group treated with a leptin concentration found in morbidly obese men (Martins, Moreira et al. 2015). Our results showed that *SIRT1* mRNA levels are increased in that same group of cells treated with a concentration found in morbidly obese men when compared with the remaining groups, which further support the findings that glycolysis is affected. SIRT1 modulates the effects of PGC-1α repression of glycolytic genes through deacetylation, which could also interfere with glycolysis, compromising even further the nutritional support of spermatogenesis, a hypothesis that remains to be tested. In addition, SIRT1 also suppresses hypoxia inducible factor 1α (HIF-1α), decreasing the rate of glycolysis and promoting oxidative metabolism (Kim, Tchernyshyov et al. 2006, Lim, Lee et al. 2010). Notably, most cancers develop hypoxia due to cellular proliferation and the response to hypoxia results in an increased tumor proliferation in a positive feedback loop. In breast cancer, hypoxia inducible factors (HIFs) are well described as factors required to promote breast cancer stem cells proliferation (Semenza 2015), which could explain why in human breast cancer cells, exposure to 50 ng/mL of leptin shifted ATP production from glycolysis to oxidative metabolism (Blanquer-Rossello, Santandreu et al. 2015). Usually associated with hypoxic conditions, HIFs activity regulated by hormones, such as insulin, under normoxic conditions has been reported in the last years (Treins, Giorgetti-Peraldi et al. 2002, Biswas, Mukherjee et al. 2013). Interestingly, the expression of two isoforms of HIFα, HIF-1α and HIF-2α, was observed in SCs (Gruber, Mathew et al. 2010, Guven, Ickin et al. 2014). In a recent study, higher HIFs transcriptional activity was associated with a higher lactate production, glucose uptake and lactate dehydrogenase activity in rSCs. Furthermore, FSH stimulation increases HIF-1α protein and *HIF-1a* mRNA levels, whereas the use of a pharmacological agent that degrades HIFα subunits inhibits FSH-stimulated lactate production and glucose uptake (Galardo, Gorga et al. 2017). These studies suggest a role for HIFs in the regulation of lactate provided to germ cells through FSH stimulation. A suppression of HIF-1α by SIRT1 would have implications in lactate production and could disrupt the development of germ cells. However, further studies are needed to confirm this assumption. SIRT1 modulation of PGC-1α does not directly regulate the effects of PGC-1α in mitochondrial genes. Since no differences were obtained in the mRNA levels of the other mitochondrial biogenesis markers studied in hSCs (Rodgers, Lerin et al. 2005), we speculate that the changes in SIRT1 suggest an involvement in a signaling pathway not directly associated with mitochondrial function. Thus, dysfunction in SIRT1-HIF1a axis induced by high levels of leptin, rather than mitochondrial biogenesis in SCs, can be a crucial mechanism to mediate the dysfunction known to occur in obese men. Indeed, no statistical differences were found in the number of mitochondrial copies between the groups treated with the different leptin concentrations used in this work and the non-treated cells, which further strengthens our hypothesis that leptin can modulate mitochondrial function instead of mitochondrial number. In fact, CII protein levels were lower in the groups treated with a leptin concentration found in lean men and with a leptin concentration found in morbidly obese men, which could interfere with OXPHOS, illustrating a way through which leptin modulates mitochondrial function. However, CII contributes less to the overall electron

transport chain process, since no protons are transported to the intermembrane space in this step of OXPHOS, suggesting that leptin does not induce a very relevant impairment on oxidative phosphorylation. Further functional studies of mitochondrial respiration in the presence of leptin and after stimulation/repression by this hormone should be performed, to confirm this hypothesis.

Obesity and its associated co-morbidities have reached pandemic proportions. The number keeps rising and men, at younger age, are suffering from subfertility and infertility. Among the several changes in the hormonal profile of these individuals, increased leptin levels are a well-known trait of obesity. Furthermore, the effects of leptin on peripheral tissues has gained increased attention in the last years, with hyperleptinemia being associated with several co-morbidities. In SCs, the effects of leptin are still undisclosed. However, the leptin receptor has recently been identified in hSCs and the direct action of this hormone on these cells showed that leptin modulates their metabolic behavior. Leptin ability to modulate mitochondria dynamics and biogenesis in several systems was also reported. Overall, our results show that leptin modulates metabolic activity and mitochondrial membrane potential in rSCs in a concentration of 50 ng/mL. This could be a novel factor contributing for mitochondrial dysfunction observed in morbidly obese individuals. Furthermore, this could also be a potential way through which leptin could affect spermatogenesis, disrupting SCs mitochondrial functionality, explaining in part, the lower fertility potential of obese and morbidly obese individuals. However, mRNA levels of several genes involved in mitochondrial biogenesis were not altered. When compared with hSCs, where a metabolic modulation by leptin was already well reported by our group, there were some important differences. For instance, *SIRT1* mRNA levels were decreased, which due to the role that SIRT1 has on glycolysis further supports that leptin affects glycolysis in hSCs, but no evidence for that was found in rSCs. These discrepancies reflect the differences that exist between the two species and the precautions that must be taken when discussing results obtained in animal models and the attempt to translate them directly to humans. Nonetheless, further studies are needed to clarify the meaningfulness of these differences.

This work presents some inherent limitations. The use of immortalized cell lines, such as the one selected to study rSCs comes with drawbacks, as these cells may not behave identically with primary cells. Furthermore, since these cells are genetically manipulated this could alter their phenotype and their responsiveness to stimuli. SerW3 rSCs is a cell line immortalized with SV40 large T-antigen, that despite showing many of the functional characteristics of SCs, is not responsive to FSH. Nevertheless, our work contributes to the knowledge concerning the action of leptin in rat and human SCs, particularly in its influence on mitochondria. Studies are needed to further clarify most of the mechanisms behind leptin's ability to modulate mitochondrial function and biogenesis, such as the study of

oxygen consumption, evaluation of oxidative stress parameters and the proteins levels involved in the mitochondrial fusion-fission cycles.

VI. Conclusion

Obesity is reaching pandemic proportions. Accompanying this trend, subfertility and infertility are poised to be one of the greatest challenges of the 21st century. Sperm parameters are reaching critical levels and the need to resort to fertility clinics among couples in reproductive age is rising all over the world. Despite being overlooked in the past decades, a relation between these two problems exists, since obesity is characterized by changes in the metabolic profile of the individual and the reproductive processes, such as spermatogenesis, are heavily dependent on the metabolic profile of the individuals and testicular cells. Thus, it urges to study the possible mechanisms that exist connecting these two topics. In order to clarify some of these mechanisms, we chose to study SCs, as these cells are responsible for the establishment of the nutritional microenvironment where germ cells develop. Furthermore, we specifically studied the influence of leptin on mitochondria. Our first approach consisted in evaluating the effects of leptin in the proliferation and metabolic activity of rSCs. Afterwards studies were performed to evaluate leptin effects in mitochondria, assessing the levels of mitochondrial complexes, mRNA levels of biogenesis markers and mitochondrial membrane potential in rSCs. Following our initial results, we decided to evaluate the effects of leptin in mitochondrial complexes and mRNA levels of biogenesis markers in hSCs. Our results show that leptin in a concentration of 50 ng/mL decreases mitochondrial membrane potential, which reflects mitochondrial dysfunction caused by leptin levels found in morbidly obese individuals. This could represent a novel mechanism through which leptin contributes to obesity-induced subfertility or infertility in males. Interestingly, leptin exposure has no effect in protein levels of mitochondrial complexes and mRNA levels of biogenesis markers in rSCs, suggesting that leptin affects mitochondrial function. Furthermore, differences were observed between rSCs and hSCs after exposure to leptin which reflects the differences that exist between the two species and the precautions that must be taken when discussing results obtained in animal models and the attempt to translate them directly to humans. In hSCs, mRNA levels of *SIRT1* presented changes in the group treated with 50 ng/mL of leptin and protein levels of mitochondrial complex II presented changes in the groups treated with 5 and 50 ng/mL, of leptin while in rSCs non-statistically significant differences were observed. These changes are species-dependent and, concerning *SIRT1* mRNA levels, may represent a novel mechanism through which leptin affects the metabolic control of spermatogenesis with possible implications in the glycolysis occurring in hSCs but not in rSCs. These changes could explain in part the decrease in sperm parameters of morbidly obese individuals. Nonetheless, further studies are needed to unveil the exact mechanisms through which leptin affects mitochondria modulating SCs metabolism. The dysfunction in leptin normal levels could compromise the nutritional support of spermatogenesis and consequently, male reproductive health.

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VIII. Annex I

List of publications resultant from the work developed during the M.Sc. in Biomedical Sciences

Moreira BP, Monteiro JP, Meneses MJ (2017) Testis Physiology. In: Alves MG, Oliveira PF (eds) Biochemistry of Andrology, vol 1. Andrology: Current and Future Developments. Bentham Science Publishers, Sharjah, UAE, pp 6-37. (ISBN: 978-1-68108-501-2)

Moreira BM, Dias AC, Monteiro MP, Sousa M, Alves MG, Oliveira PF (2017) Leptin modulates Sertoli cells mitochondrial function and biogenesis with implications for the nutritional support of spermatogenesis. Endocrine Abstracts 49: EP1179. (DOI:10.1530/endoabs.49.EP1179)

Martins AD, Moreira BM, Barros A, Sousa M, Monteiro MP, Oliveira PF, Alves MG (2017) Ghrelin, leptin and glp-1 as modulators of the nutritional support of spermatogenesis. Obesity Facts 10(S1):72. (DOI:10.1159/000468958)

Moreira BM, Dias AC, Monteiro MP, Sousa M, Alves MG, Oliveira PF. Leptin modulates Sertoli cells mitochondrial function and biogenesis with implications for the nutritional support of spermatogenesis. ECE 2017 - 19th European Congress of Endocrinology, 20 - 23 May 2017, Lisbon, Portugal.

Martins AD, Moreira BM, Barros A, Sousa M, Monteiro MP, Oliveira PF, Alves MG. Ghrelin, leptin and glp-1 as modulators of the nutritional support of spermatogenesis. ECO2017 - 24th European Congress on Obesity, 17 - 20 May 2017, Porto, Portugal.

IX. Annex II

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