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Doutor Marco G. Alves
Professora Doutora Sandra Paiva

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NOVOS BIOMARCADORES ASSOCIADOS À HERANÇA METABÓLICA DA OBESIDADE ATRAVÉS DOS GAMETAS MASCULINOS

Os genes relacionados à obesidade (ORG) têm sido apontados como causas de um fenótipo de sobrepeso e/ou obesidade, sugerindo que crianças nascidas de pais obesos possuem uma maior predisposição para desenvolver distúrbios metabólicos. Este projeto investigou mecanismos moleculares relacionados com a transmissão de um fenótipo de sobrepeso e/ou obesidade. Foi colocada a hipótese de que os ORG, recetor de melanocortina-4 (*MC4R*), gene de obesidade e de massa de gordura associada (*FTO*), glucosamina-6-fosfato deaminase 2 (*GNPDA2*) e proteína transmembranar 18 (*TMEM18*) são fatores importantes nas células de Sertoli (SCs) e espermatozoides. Propusemos também que a expressão destes ORG poderia estar relacionada com o desenvolvimento embrionário e taxa de gravidez de casais em tratamentos de reprodução medicamente assistida. Neste trabalho, identificamos a expressão de *FTO, MC4R, TMEM18* e *GNPDA2* e respetivas proteínas em SCs humanas. Identificamos ainda a expressão de *FTO*, *MC4R*, *GNPDA2* e respetivas proteínas em espermatozoides humanos. De seguida, avaliamos se a expressão de ORG nas SCs respondia a hormonas associadas à obesidade, tratando as células com doses crescentes de leptina, grelina e *glucagon-like protein* (GLP-1), mimetizando níveis hormonais relatados em indivíduos desnutridos, normais, obesos e gravemente obesos. A expressão de *GNPDA2* e *TMEM18* aumentou após tratamento das SCs com as concentrações mais elevadas de leptina e grelina, respetivamente. De seguida, investigamos se a expressão de ORG poderia estar relacionada com a qualidade espermática e o desenvolvimento do embrião. Verificamos que a idade paterna e o índice de massa corporal (BMI) não se correlacionam com a expressão de ORG em espermatozoides. No entanto, a expressão de *MC4R* e *FTO* está correlacionada com a viabilidade espermática (r= -0.3111) e contagem total de espermatozoides (r=0.5042), respetivamente. A expressão de *FTO* está também correlacionada com a qualidade do embrião, particularmente com a taxa de fertilização (r= 0.4751), a taxa de clivagem do embrião (r= 0,6530) e a taxa de embriões de alta qualidade (r= 0.6544). A expressão de *MC4R* nos espermatozoides também está correlacionada com a taxa de gravidez bioquímica (r= 0.4502), um parâmetro associado ao início do processo de implantação pelo embrião. Em suma, a abundância de ORG no espermatozoide mostrou estar associada a parâmetros relevantes da qualidade do esperma e associada ao sucesso clínico das técnicas de reprodução medicamente assistida. No entanto, mais estudos são necessários para desvendar o papel desses ORG nos espermatozoides e SCs.

Palavras-chave: Fertilidade, Metabolismo, genes relacionados à obesidade, Célula de Sertoli, qualidade espermática.

NOVEL BIOMARKERS FOR METABOLIC INHERITANCE OF OBESITY THROUGH MALE GAMETES

Obesity-related genes (ORG) have been pointed out as causes for an overweight/obese phenotype, suggesting that children born from obese parents could have a genetic predisposition to develop metabolic disorders. This project aimed to unveil molecular mechanisms related with the inheritance of an overweight/obese phenotype through sperm. We hypothesized that ORG, Melanocortin-4 receptor (*MC4R*), Fat mass and obesity (*FTO*), Glucosamine-6-phosphate deaminase 2 (*GNPDA2*), and Transmembrane protein 18 (*TMEM18*) were present and could have a role in Sertoli cells (SCs) and sperm physiology. In addition, we also proposed that ORG expression was important for embryo development, being correlated with pregnancy rates in couples seeking paternity through medical-assisted reproduction treatments.

We identified the expression of *MC4R, TMEM18, GNPDA2, FTO,* and respective proteins, in human SCs. We further identified the expression of *MC4R, GNPDA2, FTO,* and respective proteins, in human spermatozoa. First, we evaluated if ORG expression in SCs responded to hormonal dysregulation associated with obesity. SCs were treated with increasing doses of obesity-related hormones, leptin, ghrelin, and glucagon-like protein 1 (GLP-1), mimicking the hormonal levels reported in undernourished, normal, obese and severely obese individuals. The expression of *GNPDA2* and *TMEM18* was increased after exposure to the highest concentration of leptin and ghrelin, respectively. Afterward, we investigated if the abundance of ORG could be associated with the overweight/obesity effects in sperm quality and embryo development. Paternal age and body mass index were not correlated with the abundance of ORG in spermatozoa. However, the expression *MC4R* and *FTO* was correlated with sperm quality through sperm viability (r=-0.3111) and total sperm count (r=0.5042), respectively. Furthermore, the expression of *FTO* was also correlated with embryo quality, particularly with the fertilization rate (r= 0.4751), embryo cleavage rate (r= 0.6530), and high-quality embryo rate (r= 0.6544). The expression of *MC4R* in spermatozoa was also correlated with the biochemical pregnancy rate (r= 0.4502), a parameter that is associated with the initiation of the implantation process by the embryo. In sum, the abundance of ORG in spermatozoa was associated with relevant parameters of sperm quality and with the clinical success of assisted reproduction techniques. Further studies are necessary to unveil the role of these ORG in spermatozoa and Sertoli cells.

Keywords: Fertility, metabolism, Obesity-related genes, Sertoli cell, sperm quality.

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LIST OF ABBREVIATIONS

5α-Dihydrotestosterone	DHT
17β-Estradiol	E2
α-Melanocyte-stimulating hormone	α-MSH
β2-microglobulin	β2-MGB
Agouti-related protein	AgRP
Asthenozoospermia	AT
AT-rich interactive domain-containing protein 5B	ARID5B
Biochemical pregnancy rate	BP
Body mass index	BMI
Blood-testis barrier	BTB
c-Jun N-terminal kinase	JNK
Cyclic Adenosine 3',5'-monophosphate	cAMP
Fat mass and obesity gene	FTO
Follicle-stimulating hormone	FSH
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Glucagon-like protein	GLP-1
Glucosamine-6-phosphate deaminase 2	GNPDA2
Gonadotropin-releasing hormone	GnRH
Hypothalamic-pituitary-gonadal	HPG
Immunofluorescence staining	IF
Intracytoplasmic sperm injection	ICSI
In vitro fertilization	IVF
Iroquois homeobox protein 3	IRX3
Iroquois homeobox protein 5	IRX5

Lactate dehydrogenase	LDH
Luteinizing hormone	LH
Melanocortin-4 receptor	MC4R
Membrane glucose transporters	GLUTs
N^6-methyladenosine	m^6A
Normozoospermia	NZ
Obesity-related gene	ORG
Obstructive azoospermia	A
Oligoasthenoteratozoospermia	OAT
Oligospermia	O
Oligoteratozoospermia	OT
Peroxisome proliferator-activated receptor gamma	PPARG
Phosphate-buffed saline	PBS
Phosphatidylinositol 3-kinase	PI3-K
Polymerase chain reaction	PCR
Protein kinase A	PKA
Proton/Monocarboxylate transporters	MCTs
Quantitative polymerase chain reaction	qPCR
Reactive oxygen species	ROS
Sertoli cells	SCs
Sex hormone-binding globulin	SHBG
Teratozoospermia	T
Transmembrane protein 18	TMEM18
Western-blot	WB
World Health Organization	WHO

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Chapter I

INTRODUCTION AND AIMS

1. INTRODUCTION

1.1. The decline of fertility in modern societies

A large diversity of living things cohabitate on earth and all sustain their evolutive success through reproduction. It is not only relevant to enhance the chances of survival of the species but also to ensure continuity and propagation. However, it is well-known that fertility rates are decreasing worldwide. Several authors had discussed this phenomenon, but the reasons behind it are complex and difficult to unveil and thus, a consensus has not been reached yet. The anthropologist Marvin Harris proposed, in 1989, that people adjust their fertility to the economic value of children's labor, explaining why fertility rates were higher in developing countries at the time (1). Several recent works highlight that the increased incidence of metabolic diseases, known to induce alterations in the male and female reproductive tracts, is associated with the reported decrease in birth rates worldwide. This subject has been overlooked for years but is now clear that deserves special attention from researchers, stakeholders, media, and politicians.

Infertility, by definition, describes the inability to conceive children after 12 or more months of regular unprotected sex and it has been recognized by the World Health Organization (WHO) as a major health problem that affects individuals on both, modern and in developing societies. In a more accurate medical context, infertility is a disease of the reproductive system that prevents a sexually active couple to achieve a successful pregnancy in a period of 12 months of unprotected sex (2). Nowadays, it is assumed that infertility affects over 72 million people. However, due to the difficulty of evaluating infertility rates in developing countries, the number of affected people is must certain underestimated (3). In addition, the contribution of the male factor alone or in combination with the female factor is expected to account for almost 2/3 of the infertility cases (4).

Although medical care has substantially increased in modern society, the incidence of metabolic diseases keeps rising worldwide at a worrying level. Like other noncommunicable diseases, metabolic disorders are largely caused by lifestyle, mainly through poor dietary choices and lack of physical activity. Several authors also consider the rise in metabolic diseases as a consequence of the technologic, economic and social advances accomplished in the last decades. As human populations became more urban, carbohydrate and fiber-rich diets were replaced by sugar and fat-rich diets, while a sedentary lifestyle is promoted. In addition, most of these disorders have a strong

genetic component and are usually associated with inborn errors that could be inherited by future generations (5).

One of the most common metabolic disorders is obesity that is commonly defined by an excess of fat accumulation, resulting from a positive balance between caloric intake and energy expenditure. Even though the consumption of high-fat diets and sedentary lifestyle are major factors for the accumulation of excess weight, recent studies provided clear evidence that the development of obesity is also strongly influenced by genetics (6). Nowadays, the obesity epidemic has reached alarming proportions. According to the WHO, in 2016, more than 1.9 billion adults and 41 million children were overweight or obese (7) and these statistics can become even more serious in the years to come. It is well known that an increased body mass index (BMI) has detrimental effects in all systems of the body and is associated with the development of cardiovascular diseases, cancer, musculoskeletal disorders, diabetes mellitus, and other health complications, such as infertility (8). The growing rates of obesity incidence, along with comorbidities associated are concurrent with the serious concerns on the reproductive health of the individuals in modern societies. Nowadays, at least 1 in 7 couples will be affected by infertility (9) and the fertility rates are expected to keep falling as the number of obese individuals at reproductive age rise. Indeed, the metabolic abnormalities caused by obesity are associated with polycystic ovary syndrome and anovulatory infertility in women (9). Unsurprisingly, 50% of the women diagnosed with polycystic ovary syndrome are overweight or obese, supporting that obesity plays a pathogenic role in the development of the syndrome (10). Moreover, obese women are known to develop more pregnancy complications and to be more prone to suffer more miscarriages in the first trimester of pregnancy that normal-weight pregnant women (11). Thus, there are several clinical, epidemiologic and molecular studies showing a clear relationship between overweight/obesity and less fertility in females and a lower probability to conceive (12). Nevertheless, only recently has emerged the concept of subfertility or infertility caused by overweight or obesity in the male. Indeed, the increased incidence of male obesity is being coincident with the increase in the cases of subfertility and/or infertility in males from westernized countries. Moreover, recent studies showed that obesity in the male partner may equally impair embryo development as when occurring in the female partner (13, 14). Of note, overweight men are usually associated with lower reproductive potential and more likely to be oligospermic (a condition where semen has a very low concentration of sperm), or azoospermic (a condition where semen has no sperm) when compared to males with normal BMI values (15). These individuals are also more likely to suffer from mechanical

dysfunctions in the reproductive tract, including erectile dysfunction (16). Although many studies have associated the infertility epidemic of the couple with the increasing number of overweight and obese men, the molecular mechanisms by which spermatogenesis or sperm quality are impaired are only starting to be unveiled.

1.2. Spermatogenesis and spermiogenesis at brief

Spermatogenesis is the process where male germ cells are formed. It occurs in the testis and can be divided into three crucial phenomena: differentiation of spermatogonial stem cells into spermatocytes via mitosis, production of haploid spermatids from primary spermatocytes via meiosis and spermiogenesis (17). Sertoli cells (SCs) are the main mediators of this process and are responsible for limiting the expansion of the spermatogonial population, providing critical factors necessary for the success of spermatogenesis (18). These cells are also responsible for the formation of the blood-testis barrier (BTB), which is a structured biological barrier formed by tightly adjacent SCs that control the intratubular environment while also providing protection against the host immune system (19). The maintenance of the seminiferous tubule is achieved by a balance between germ cell apoptosis and regeneration. These processes are controlled by a complex network of endocrine and paracrine signals. SCs have receptors to follicle-stimulating hormone (FSH) and testosterone, the main hormonal regulators of spermatogenesis, both acting as germ cell survival factors (18). These hormones regulate glucose uptake and lactate production by SCs (20). Sertoli and germ cells communicate by both paracrine signals and direct membrane contact, for example, through the c-kit receptor, expressed on the surface of spermatogonia, and stem cell factor, produce by SCs (18).

Spermatogonia and SCs adhere to the basement membrane of the seminiferous tubule. In humans, it is possible to distinguish three types of spermatogonium: type A (dark) are reserve spermatogonia cells and do not undergo mitosis and type A (pale) undergo through mitotic division to give rise to type B (19). Under the correct conditions, each type B spermatogonia undergoes through one mitotic division, producing primary spermatocytes. After having completed meiosis I, primary spermatocytes give rise to secondary spermatocytes. When meiosis is completed, haploid spermatids are formed (17, 21) (**Figure 1**).

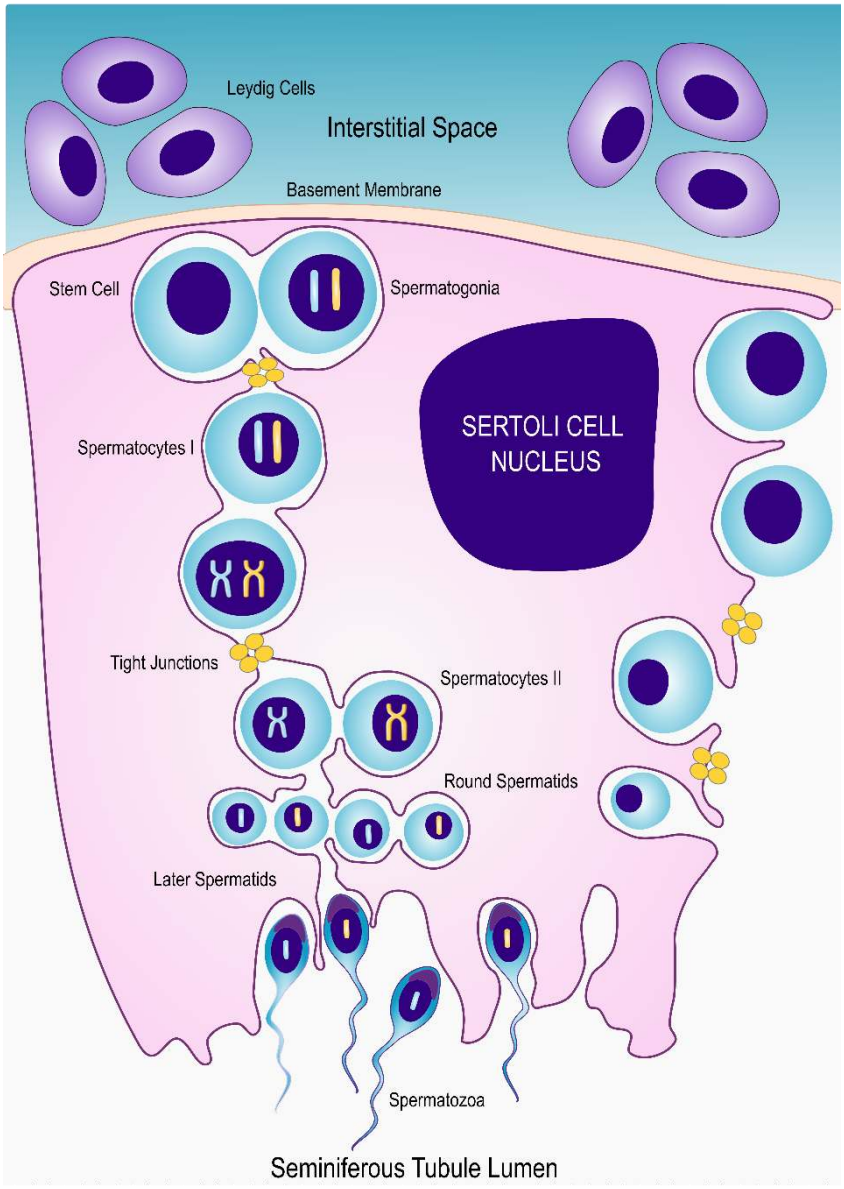

Figure 1: Schematic representation of the interaction between Sertoli cells and germ cells, during spermatogenesis. The SCs are located at the base of seminiferous tubule and provide support for the developing spermatogonia. SCs are responsible for the limitation of the expansion of the spermatogonial population providing critical factors necessary for the success of spermatogenesis. Under the right conditions, diploid spermatogonia undergo through meiotic division to form haploid spermatids. Finally, spermatids undergo cytodifferentiation to form spermatozoa. [Adapted from: "Blood-Testis Barrier: How Does the Seminiferous Epithelium Feed the Developing Germ Cells?" (23)]

Spermiogenesis can be described as a series of cytodifferentiation that spermatids undergo to form spermatozoa capable of motility (22). At the beginning of this process, spermatids have a round shape and central nucleus. Spermiogenesis starts with nuclear condensation and migration of the

nucleus to the periphery of the cell. Meanwhile, the acrosome is assembled, which is a Golgi-derived organelle that can be described as a specialized lysosome, essential for the interaction with the egg during fertilization, and microtube-based axoneme, essential for mobility (19, 22). The latter will become the central structure of the flagellum, which is assembled soon after meiosis is completed. Following these events, spermatids shed a large part of its cytoplasm and the elongated spermatids are released from the seminiferous epithelium. This last event is called spermiation.
Yet, spermatozoa released into the seminiferous tubules lumen are still not able to move. The seminiferous tubules converge into the epididymis, tubular structures behind the testis where sperm maturation occurs. However, the exact molecular process behind sperm maturation is largely unknown (24). In humans, the process of formation and maturation of spermatozoa takes around 74 days (22).

1.3. Sertoli Cells, the sentinels of spermatogenesis

SCs, also known as nurse cells, are responsible for fulfilling the physical and nutritional needs of the developing germ cells. Each SCs has the ability to support around 30 to 50 developing germ cells. These cells are characterized by a very complex cup-structure. This three-dimensional structure is constantly changing, allowing SCs to interact with the developing germ cells and the epithelium cells of the seminiferous tubules (25). Mature SCs produce extracellular matrix components, such as collagen and laminin, to form specialized junctions and guarantee the maintenance of the seminiferous epithelium (25). Concurrently, SCs secret a great panoply of specific products, which are essential for the developing germ cells. The transference of these products can only be accomplished due to the close relationship between germ cells and SCs. Moreover, adjacent SCs form tight junctions, creating the BTB (26). These SCs characteristics allow them to control the intratubular environment. Since developing germ cells are unable to use glucose as an energy substrate, SCs convert most of the glucose into lactate, which can then be used by germ cells as an energy substrate. The uptake of glucose from the interstitial space by the SCs occurs via specific integral membrane glucose transporters (GLUTs) being that GLUT1 and GLUT3 appear to have the most relevant role regarding SCs metabolism (27), though GLUT2 appears to be highly expressed (28). The conversion of glucose into lactate is divided into two fundamental steps. In the first step, glucose is metabolized into pyruvate. Pyruvate is then converted into lactate by lactate dehydrogenase (LDH), synchronously with the oxidation/reduction of NADH to NAD^+. Finally, lactate is exported into the intratubular fluid of the seminiferous tubules

by specific proton/monocarboxylate transporter 4 (MCT4). Herein, lactate becomes available to the developing germ cells, where it is captured via specific monocarboxylate transporter 1 and 2 (MCT1 and MCT2) (29). Alternatively, pyruvate can also be converted into alanine via alanine transaminase or be transported to the mitochondria matrix, where it is converted in acetyl-CoA and directed to the tricarboxylic acid cycle. Furthermore, acetate is also produced at high rates by SCs. Although its role remains to be further elucidated, acetate is an intermediate for the synthesis of fatty acids and appears to be related to the high demand of lipids needed by the developing germ cells (**Figure 2**).

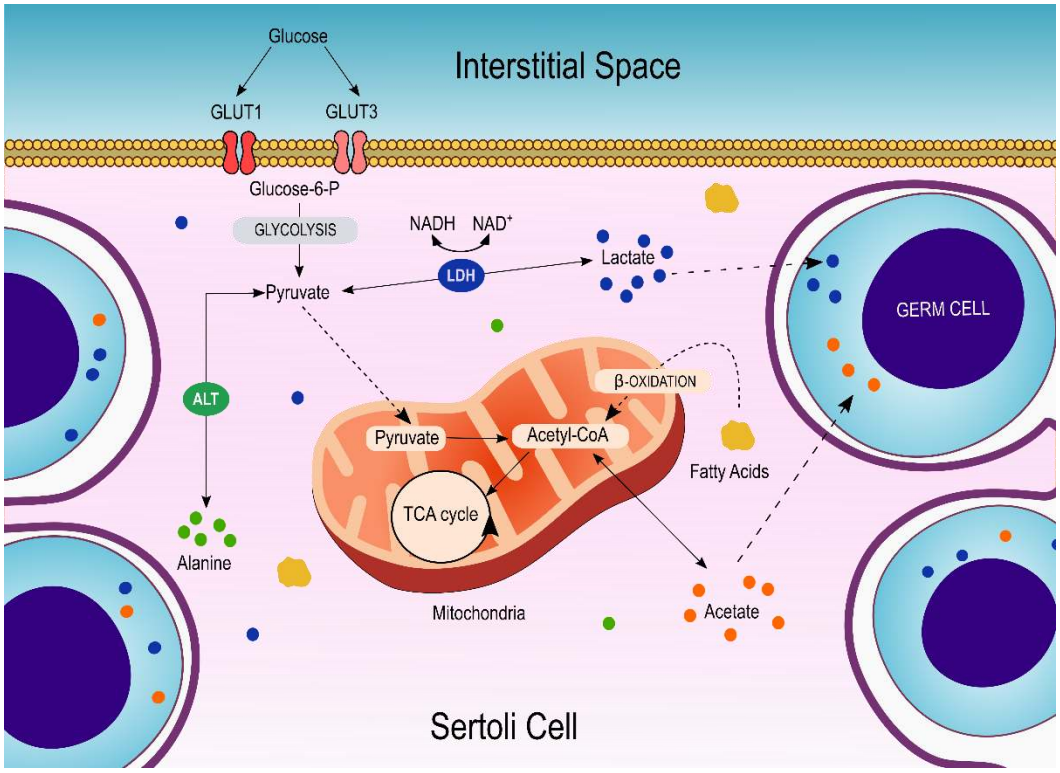

Figure 2: Schematic representation of Sertoli Cell metabolism and its main metabolic pathways. Glucose is converted into pyruvate by glycolysis. Pyruvate can participate in several metabolic pathways. In these cells, the majority of pyruvate is converted into lactate by LDH, which is then exported into the intratubular fluid and captured by germ cells through proton/monocarboxylate transporters. Alanine transaminase (ALT) promotes the convention of pyruvate into alanine which, acts as an energy substrate reservoir. Alanine can again be converted into pyruvate through alanine transamination. On the mitochondria, acetyl-CoA fuels the tricarboxylic acid (TCA) cycle, responsible for energy production. Finally, acetyl-CoA can also be converted into acetate which appears to be an important intermediate of fatty acids synthesis. The transport of metabolites is represented by the dashed lines.

The high metabolic plasticity of SCs allows them to metabolize other substrates in addition to glucose such as ketone bodies, fatty acids, and glycogen. These characteristics allow SCs to ensure metabolites production even in glucose privation situations (30).

1.4. Hormonal modulators of the male reproductive system

Due to the importance of glucose to the maintenance of spermatogenesis, the energy state of the organism can induce several alterations in the reproductive system. The glucose metabolism in SCs and the reproductive events are mainly controlled by the endocrine system, predominantly by FSH, sex steroid hormones, and insulin. The hypothalamic-pituitary-gonadal (HPG) axis is responsible for the coordination of reproductive events (**Figure 3**). The hormonal messengers associated with this axis can interact not only with the developing germ cells but, also with their caretakers. The gonadotropin-releasing hormone (GnRH) is produced by the hypothalamus and stimulates the production of the pituitary hormones, FSH and luteinizing hormone (LH) (31). In the seminiferous tubules, the SCs are the only cells that possess FSH and testosterone receptors. Nowadays, it is described that FSH can activate at least 5 signaling pathways on SCs. The crosstalk between them determines the final cellular consequence. Among the 5 known signaling pathways, the cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase (PKA) pathway, the mitogen-activated protein kinase (MAPK) pathway, and the phosphatidylinositol 3-kinase (PI3-K) pathway are the most well studied (31). The cAMP-PKA pathway was the first to be identified. Herein, the activation of the FSH receptor culminates with the expression of various isoforms of cAMP-responsive element modulator, which are required for spermatocytes and spermatids survival (32). The stimulation of the MAPK pathway by FSH is responsible for the proliferation of the SCs and occurs in a stipulated period of 15 days postpartum, in humans. The number of SCs remains relatively constant throughout adulthood, approximately 3,700 million cells per testis. The activation of the PI3-K pathways by FSH was first described in granulosa cells (33). The increase of cAMP levels mediated by FSH-receptor activation promotes the activation of PI3-K, which is a key enzyme involved in several biological responses, such as mitogenesis and glucose uptake (34). On SCs, PI3-K promotes the uptake of glucose and promotes transferrin production, both essential for the maintenance of spermatogenesis. Moreover, androgen aromatization is stimulated by SCs and germ cells by the phospholipase A2 pathway (35).

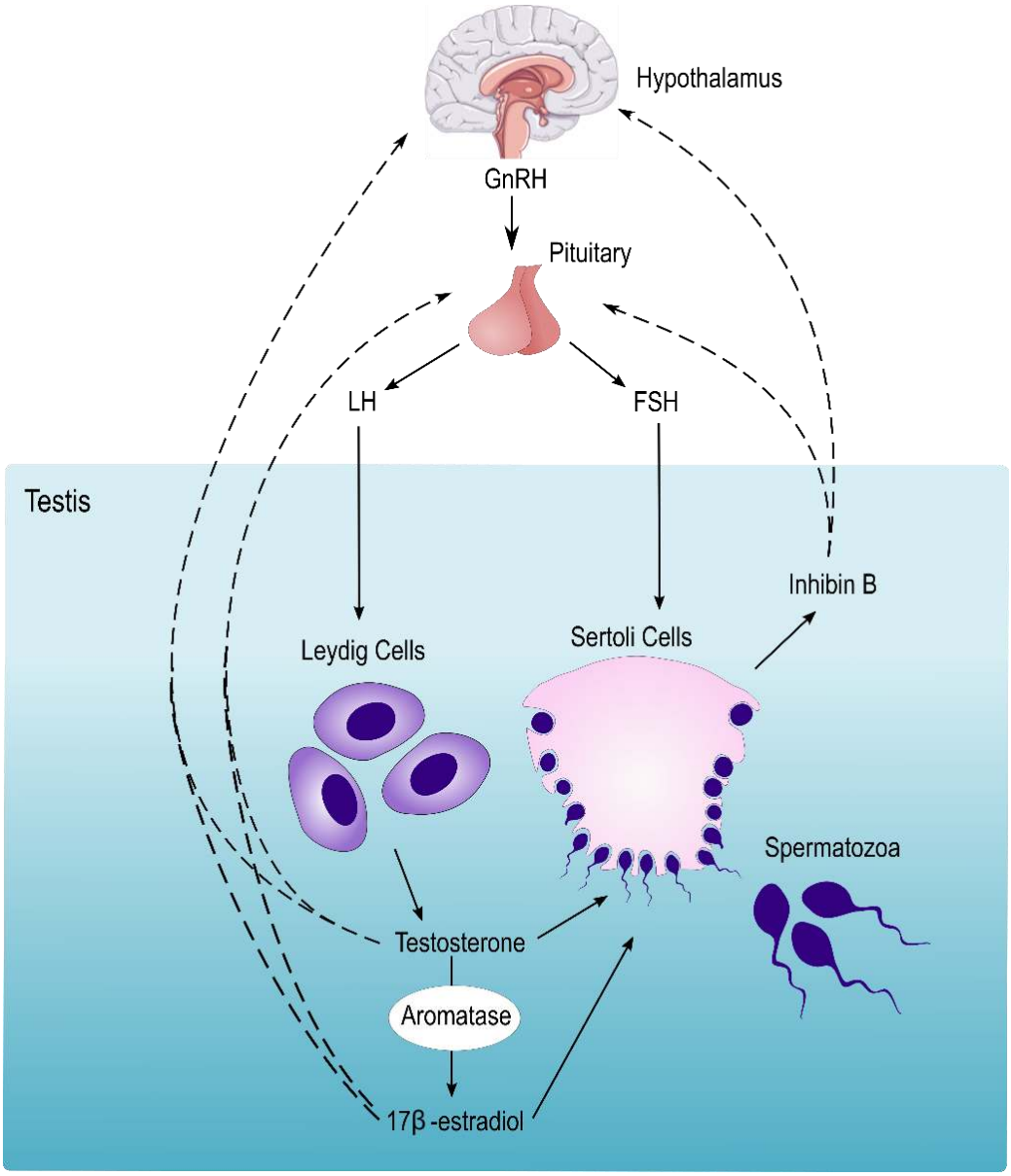

Figure 3: Schematic representation of the male Hypothalamus-Pituitary-Gonadal axis. GnRH stimuli on the pituitary promote the synthesis of gonadotropin hormones LH and FSH. While LH will promote steroidogenesis in LCs, FSH promotes spermatogenesis through the stimulation of SCs. As a result, LCs produce testosterone, a crucial regulator of spermatogenesis, while SCs produce inhibin B. A small part of testosterone suffers aromatization by testicular aromatase, producing 17β-Estradiol. These hormones promote a negative feedback loop between the testis, hypothalamus, and pituitary (represented by the dashed lines).

Testosterone produced by Leydig cells (LCs), after LH stimulation, can interact with SCs by the androgen receptor. Although FSH and testosterone display redundant functions on SCs, there are some distinct differences between the action of these hormones. First, testosterone is able to

activate the MAPK, CREB phosphorylation and CREB-mediated transport of SCs without the up-regulation of cAMP (36). This may explain how testosterone, but not FSH, can maintain spermatogenesis independently, suggesting the cAMP is not essential for spermatogenesis. Moreover, the expression of some genes is exclusively regulated by the interaction between DNA and the androgen receptor, where testosterone stimulation is required (37). The action of testosterone through the non-classical pathway promotes the attachment of germ cells to SCs and promotes the reformation of the BTB (38). In its turn, the SCs produce inhibin B, which, along with testosterone and 17β-estradiol (E2), provides a negative loop that reduces the expression of GnRH by the hypothalamus (39).

The sex steroid hormones 5α-dihydrotestosterone (DHT), mainly produced on the prostate by 5α-reductase, and E2 were reported to modulate glucose consumption and lactate production in SCs. DHT increase glucose consumption without increasing lactate production, suggesting it stimulates the Krebs cycle. This hypothesis proposes that DHT can metabolically modulate SCs to a more efficient energy status (40). Alternatively, E2 does not seem to interfere with lactate production in SCs (40). However, *in vitro* human SCs treated with E2 demonstrated increased production of acetate (41). This metabolite appears to play a key role in the progression of spermatogenesis, particularly as a precursor of cellular constituents' synthesis (41). Furthermore, both DHT and E2 are able to downregulate the transcript levels of glucose transporters, GLUT1 and GLUT2 after a 50h treatment on *in vitro* human SCs. These results appear to describe a metabolic shift from an exponential phase of glucose consumption to a stationary phase (42).

Insulin is a hormone produced by the islets of Langerhans of the pancreas and a powerful modulator of SCs metabolism (43). The insulin receptor was identified in rat SCs by OonK RB and colleagues suggesting that insulin had specific functions in the maturation and activity of these cells (44). But many studies showed new functions for this hormone in these cells. For instance, insulin-deprived SCs also presented decreased transcript levels of genes associated with lactate metabolism (*LDH* and *MCT4*) (45). In addition, those insulin-deprived cells present decreased glucose consumption and acetate production (41). Although all these *in vitro* results need further clarifications, insulin appears to be an important regulator of SCs metabolism. These results suggest that insulin dysregulation could be one of the main causes of infertility in diabetic men (46). More recently, other hormones associated with the energy homeostasis and appetite regulation were reported as metabolic modulators of SCs.

1.4.1. Leptin

Leptin is a peptide hormone produced mainly by white adipocytes, which is related to adipose tissue mass and decrease of food intake (47). The action of this hormone is modulated by the membrane-spanning leptin (or obesity) receptor (48). The hypothalamus is suggested as the primary target of this hormone. Leptin, by itself, is not capable to lead to the termination of a meal but it can interact with other hypothalamic hormone pathways associated with appetite-regulation (47, 49). In nutrient deprivation situations, the decreased plasma leptin levels induce neuroendocrine responses to food restrictions. The hypothalamic neurons express neuropeptide Y and agouti-related protein (AgRP) (47). The first acts on Y1 and Y5 receptors and stimulates food intake by increasing motivation to eat, delaying satiety and augmenting meal size (50). The AgRP is an antagonist to the melanocortin-4 receptor (MC4R) and increases food intake by antagonizing the effect of the α-melanocyte-stimulating hormone (α-MSH) (51). Under the stimuli of leptin, pro-opiomelanocortin neurons express cocaine and amphetamine-related peptide and α-MSH. These peptides induce the decrease of food intake by promoting short-term satiety (49) and interacting with other appetite-regulation pathways, including the endocannabinoid system (52).

The actions of leptin in the hypothalamus were suggested to act as modulators of the reproductive axis (53). Leptin is able to increase LH and FSH secretion by stimulation of growth and differentiation of pituitary cells, regardless of the presence or absence of GnRH (54). Nonetheless, studies have suggested that leptin mediates the HPG axis by regulating kisspeptins produced by the Kiss1 gene (55). However, the mechanisms by which leptin interacts with the HPG axis is complex and combines stimulatory and inhibitory effects. Leptin can also interact directly with the testis (56). In fact, leptin can also induce a decrease in testosterone expression by inhibiting expression of the cAMP-stimulated steroidogenic acute regulatory protein and interfering with cAMP signaling (57). Martins AD and other members from our group were the first to clearly demonstrate the presence of leptin receptor in human SCs and that leptin could directly act on these cells (28). The authors reported that leptin concentration found in lean men induce an increase of GLUT2 protein level and LDH activity, in SCs. These results suggested that leptin is a hormonal regulator of the nutritional support of spermatogenesis since all concentrations of leptin tested decrease human SCs acetate production (28) (**Figure 4**).

1.4.2. Ghrelin

Ghrelin, known as the hunger hormone, is another peptide involved in a large group of physiological functions, including food intake, sleep, body weight, inflammation and others (58). It is known that ghrelin promotes food intake both in rodents and in humans and reduces insulin secretion (59). In what concerns the reproductive system, ghrelin appears to be an important integrator of energy homeostasis control. It was proposed that ghrelin can inhibit the proliferative activity of immature germ cells and LCs (60), avoiding excess build-up of germ cells which is crucial for spermatogonia survival (61). Furthermore, recent *in vitro* studies have reported that ghrelin is able to inhibit testicular secretion of testosterone (62) while suppressing LH and follicle-stimulating hormone FSH secretion by the pituitary (63), events that could also promote the apoptosis of germ cells.

The presence of the growth hormone secretagogue receptor on human SCs was confirmed by immunohistochemistry technique (64). Martins AD and other members from our group demonstrated that ghrelin could modulate human SCs metabolic phenotype, decreasing the glucose consumption and mitochondrial membrane potential. Curiously, LDH activity and lactate production remain unaltered. These results suggest that ghrelin can act as an energy sensor for human SCs in a dose-dependent manner (65) (**Figure 4**).

1.4.3. Glucagon-like protein 1

Glucagon-like protein 1 (GLP-1) is a hormonal derived from proglucagon gene, mainly secreted by endocrine L-cells from intestines (66). Along with leptin and ghrelin, GLP-1 is one of the prominent players in glucose homeostasis, gastrointestinal motility, and appetite. The release of GLP-1 is stimulated by the presence of nutrients (predominantly sugars and fats) in the stomach and proximal intestine or through direct contact between nutrients and L-cells. GLP-1 diffuses through the capillaries and lymph. Herein, it mediates the activation of the GLP-1 receptor, present throughout the periphery (pancreas, stomach, adipose tissue, heart, and others) (67) and the central nervous system (68). The endogenous role of GLP-1 in the regulation of appetite is a concept well accepted. This hormone can decrease gastric emptying and intestinal motility. Meanwhile, the absorption and digestion of nutrients are optimized. In the pancreas, it stimulates the secretion of insulin, proliferation of islet, coupled with inhibition of glucagon secretion. The stimulation of the hypothalamus by GLP-1 induces satiety and decreases in caloric intake (69). Due to these proprieties, GLP-1 analogs are prescribed for the treatment of diabetes, a condition characterized, in a simple sum, by hyperglycemia and insulin resistance (70).

The expression of GLP-1 receptor was first identified in isolated human SCs by Martins AD and colleagues (71), which reported that exposure to increasing doses of GLP-1 did not alter the expression of this receptor. The authors reported that exposure to GLP-1 decreased glucose consumption while increasing lactate production in human SCs (**Figure 4**). Meanwhile, no effects were detected in mitochondria functionality in human SCs exposed to the GLP-1 levels found in healthy individuals.

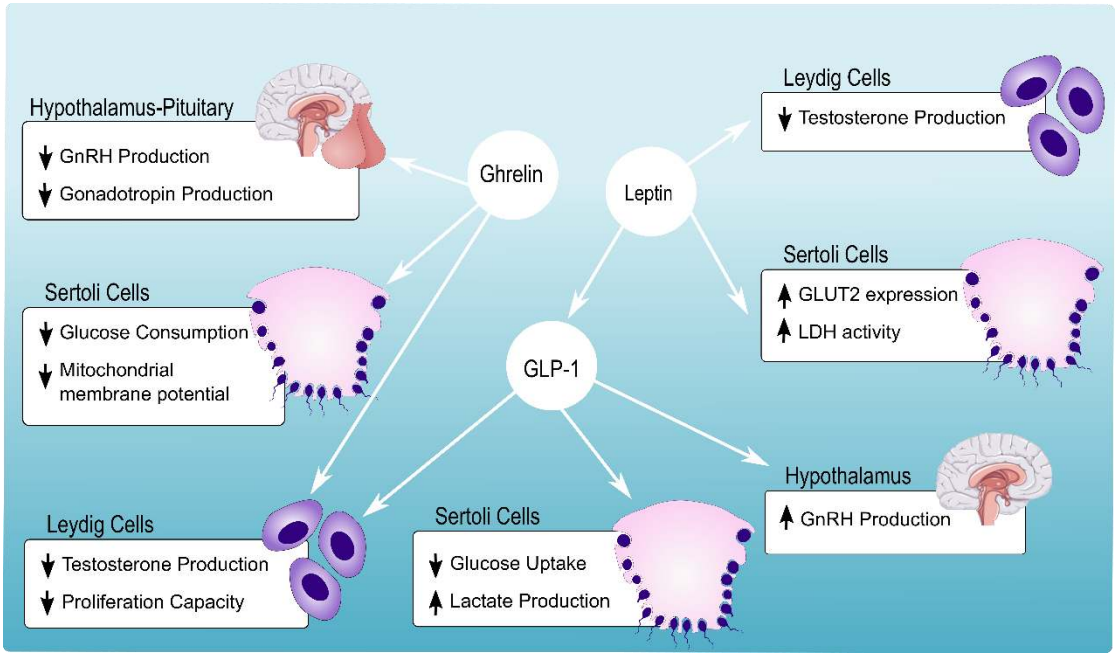

Figure 4: Schematic resume of obesity-related hormones effects on the male reproductive tract. In the pituitary, Ghrelin suppresses LH and FSH secretion leading to the inhibition of testosterone secretion. Ghrelin levels are related to decreased glucose consumption and decreased mitochondrial membrane potential of SCs, in a dose-dependent manner. Androgens are an important regulator of leptin secretion. LCs are known to express leptin receptors, which promote the reduction of testosterone secretion, upon high circulating leptin levels. Leptin also decreases SCs acetate production suggesting that it has a direct action on SCs mitochondrial function. Furthermore, increased levels of GLUT2 expression and LDH activity are also associated with leptin. It can also stimulate GLP-1 secretion, in a dose-dependent manner. In its turn, GLP-1 can interact with the HPG axis, promoting the secretion of GnRH by the hypothalamus. In LCs, it can decrease the secretion of testosterone. While on SCs, it promotes lactate production, even though glucose consumption is decreased. Down arrows stand for downregulation and up arrows stand for upregulation.

The stimuli of GLP-1 seem to be vital for eliciting the production of lactate by SCs while, consuming lower amounts of glucose. These results illustrate the metabolic plasticity that characterizes SCs. Furthermore, the authors proposed that that GLP-1 modulates the glucose metabolism of SCs and, it may have an anti-apoptotic effect in the developing germ cells (71). Additionally, this hormone is able to decrease testosterone secretion (72), suggesting that it may affect the HPG axis. Further, this hormone seems to increase GnRH secretion, which suggests that it may have an important role in puberty development (73).

1.5. Obesity and Male Infertility: Is there a link?

Obesity results from a disturbance between energy intake and expenditure. From this point of view, it is easy to associate excess of weight and obesity as the result of lack of exercise, a sedentary lifestyle and the consumption of diets rich in fat. However, the origin of obesity is multifactorial and complex. Several of those factors are very difficult to unveil, and for instance, the development of obesity has an evident environmental contribution. Indeed, some environmental compounds are classified as obesogens, a class of chemical compounds that can enhance adipogenesis and promote lipid accumulation. The exposure to these compounds, usually through diet, is known to impair energy metabolic pathways and to disrupt signaling pathways that control food intake (74). Obesogens are reported to have great stability and be structurally similar to androgens. Further, a large portion of these compounds is lipophilic, which means that they can disrupt the endocrine system. These effects will be reflected in the reproductive systems of both males and females (75-77). However, the impact of obesity on the fertility potential of males has been disregarded for several decades. In addition, previous studies focused on obesity and classic sperm parameters (namely, concentration, motility, and morphology) have reported mixed results (summarized in **Table 1**).

While several studies showed that excess weight is related to low semen volume and sperm motility as well as aberrant sperm morphology (99-101), other studies found no significant correlation (79, 80, 102). The observed discrepancies in the literature are likely caused by the several limitations associated with studies performed in humans. In addition, sperm function can be altered by innumerable lifestyle factors, such as smoking, alcohol consumption, and recreational drugs, which are confounding factors difficult to control in human studies.

Table 1: Summary of studies investigating the effects of obesity on classic sperm parameters.

Reference	Concentration	Motility	Normal Morphology
Jensen, Andersson (78)	↓	No correlation	↓
Pauli, Legro (79)	No correlation	No correlation	No correlation
Chavarro, Toth (80)	No correlation	No correlation	No correlation
Hofny, Ali (81)	↓	↓	↓
Martini, Tissera (82)	No correlation	↓	No correlation
Paasch, Grunewald (83)	↓	No correlation	↓
Sekhavat and Moein (84)	↓	↓	No correlation
Wegner, Clifford (85)	No correlation	No correlation	No correlation
Rybar, Kopecka (86)	No correlation	No correlation	No correlation
Shayeb, Harrild (87)	No correlation	No correlation	↓
Hammiche, Laven (88)	↓	↓	n/a
Anifandis, Dafopoulos (89)	No correlation	↓	n/a
MacDonald, Stewart (90)	No correlation	No correlation	↑
Belloc, Cohen-Bacrie (91)	↓	↓	No correlation
M. Al-Ali, Gutschi (92)	No correlation	No correlation	No correlation
Thomsen, Humaidan (93)	No correlation	No correlation	n/a
Luque, Tissera (94)	↓	↓	↓
Tang, Zhuang (95)	No correlation	↓	No correlation
Tsao, Liu (96)	↓	No correlation	↓
Oliveira, Petersen (97)	↓	↓	↓
Calderón, Hevia (98)	↓	↓	No correlation

Furthermore, the limitations of the BMI evaluation as an adiposity calculator can also be questioned (103). Obesity is also associated with the development of other complications, such as diabetes and oxidative stress, which can also impair sperm parameters (104, 105), promote sperm DNA fragmentation (80, 106, 107) and induce aberrant sperm mitochondrial function (106, 108). Beyond weight gain and adiposity, there are other relevant factors associated with obesity impact on sperm quality, including hormonal dysfunction, fat accumulation in the male reproductive tract,

accumulation of environmental toxic substances or even inflammation and oxidative stress. Below we briefly discuss each of these factors.

1.5.1. Hormonal dysregulation

Obesity causes whole body strong hormonal dysregulation. Due to the essential role of hormones in the maintenance of glucose homeostasis, the hormonal disruption affects not only the signaling pathways regarding food intake control but also other functions intimately associated with energy homeostasis, such as reproduction (109). In humans, leptin and sex hormones blood concentrations appear to be strongly related (110). Usually, androgens suppress leptin production (110), supporting that testosterone is an important regulator of leptin. Concurrently, leptin has been found to exert important effects on gonadal organs through leptin receptors that were reported in testicular cells, including in the surface of LCs (110, 111) and SCs (28). Obesity is associated with leptin resistance (112, 113), resulting in increased leptin levels as a response to increased adiposity. Besides, leptin resistance is associated with a lack of responsiveness to leptin's appetite-suppressing effects (114). Fortunately, leptin resistance is reportedly reversible since the reduction of food intake and body weight allow peripherical leptin to more easily access its hypothalamic sites (115). The excess of circulating leptin, found in obese men, appears to contribute to reducing testosterone concentrations due to impairment of LCs (116). Further, high levels of leptin are known to modulate SCs metabolism, decreasing lactate dehydrogenase activity (28) and both events have the potential to compromise male fertility. Concordantly, higher serum leptin levels found in obese infertile men were correlated with abnormal sperm morphology (81).

Contrarily to leptin, ghrelin resistance has not been associated with obesity (117). It was reported a significantly lower basal level of ghrelin in obese subjects in comparison to lean subjects. Even though the mechanism(s) underlying the decreased ghrelin levels associated with obesity remain unclear, it was hypothesized that ghrelin acts as a counter-regulatory hormone that limits energy intake in obese people (118). On the male reproductive tract, Martins AD and colleagues suggested that ghrelin can act as an energy sensor for human SCs in a dose-dependent manner, modulating the nutritional support of spermatogenesis (65). This hypothesis also supports that ghrelin balance can contribute to the metabolic dynamics within the male reproductive axis in situations of energy deficit (63, 109). Consequently, ghrelin balance disruption can induce severe complications upon the metabolism of the testis.

The incretin effect, a process that describes the increase of insulin plasma levels along with the decrease of glucagon levels, after a meal, is also impaired in obesity (119). This evidence suggests that incretin hormones, such as GLP-1, may be altered in obese subjects. However, the findings regarding GLP-1 levels in obesity were inconsistent and its variations have not been conclusively determined (119, 120). Meanwhile, some evidence suggested that leptin may exert a regulatory effect on GLP-1 secretion and that impairment of leptin regulation associated with obesity may also be associated with impairment of GLP-1 regulation (121). Although the effects of GLP-1 in male reproduction remain overlooked, its contribution to glucose homeostasis suggests that it may be important for the regulation of spermatogenesis (71).

Hyperglycemia, which is usually found in obese men, seems to cause a decrease in sex hormone-binding globulin (SHBG) production by the liver (122). SHBG is responsible for the transport of sex hormones through the blood to the target tissue. Naturally, with a decrease of SHBG concentration, testosterone availability will also decrease. Hyperglycemia per se has long been associated with compromised male infertility. The frequency and duration of hyper- and hypoglycemic events, the degree of glucose control, age and other factors are important to evaluate the occurrence of infertility (123). However, it has been reported that poor glucose control, such as in diabetic men, is associated with impairment of sperm motility (124), sperm DNA fragmentation (125) and hormonal dysregulation (126). Additionally, gonadotropins secretion tends to progressively decrease in obese men (111). Overweight is also associated with increased androgen aromatization by the adipose tissue. This may result in a decrease of total and free testosterone in serum, a condition known as hypogonadism (127).

1.5.2. Fat accumulation on the scrotum

Obese individuals have an excess and abnormally distributed fat in the testis, a condition known as scrotal lipomatosis, also associated with infertility (128). The abnormal distribution of scrotal fat can form a diffuse sheet of fat with variable thickness and diffuse fat covers the cord veins and the spermatic cord. Additionally, a lobular pattern of fat distribution can be found in the internal spermatic fascial tube (128). One of the main reasons why lipomatosis induces infertility is the impairment of thermoregulation in the testis of those individuals. Ideally, the scrotal sac is composed of thin skin with minimal subcutaneous fat, dense sweat glands, and scant hair distribution. These characteristics are essential for testicular aeration and heat radiation dispersion. Furthermore, vasodilation of the scrotal vessels and activation of the sweat glands are crucial for

the maintenance of the testicular temperature 2-4º C lower than the body temperature (129). Excess scrotal fat increases insulation, rising scrotal temperature and promoting testicular germinal atrophy and spermatogenic arrest. The reason behind germ cells' vulnerability to heat stress lies in their higher mitotic activity. Specifically, hyperthermic testis induces germ cell apoptosis (130), autophagy (131), DNA damage and generation of reactive oxygen species (ROS) (132). The excess of fat can compress the cord veins of the testis, being the cause of testicular ischemia, a condition characterized by the increased tension within the testis. Furthermore, the compromise blood pumping results in a venous status of the testis and testicular congestion (128) which, translates in metabolic impairment of the gonads.

1.5.3. Accumulation of environmental toxic substances

Some environmental toxins, usually agriculture pesticides or industrial compounds, act as endocrine disruptors (133). Indeed, some evidence suggests that obese individuals are at higher risk to suffer from the action of these disruptors since a large portion of them are liposoluble (134) and tend to accumulate in fat. However, the interaction between adipose tissue, environmental toxins, and male fertility remains to be elucidated. Still, the presence of organochlorines, a class of toxins usually found in pesticides (135), has already been reported in the seminal fluid of men. Some studies have already evaluated the impact of these compounds on sperm quality parameters. The exposure to some organochlorines is associated with decreased sperm counts (136), while aromatic hydrocarbons, another class of toxins, are reportedly described as major contributors for the dysfunction of sperm parameters (137). Although the presence of liposoluble toxins in the testis is likely one of the ways by which obesity induces infertility in males, this topic has been overlooked over the years.

1.5.4. Inflammation and Oxidative stress

The balance between death/regeneration of developing germ cells is essential for the maintenance of spermatogenesis. ROS are important modulators of several apoptotic signaling pathways, including the p38 MAPK pathway. Damaged germ cells produce ROS, which can then activate the p38 MAPK pathway thus starting the apoptotic process (138). This process highlights the importance of ROS in the regulation of testicular germ cell population under stress conditions. ROS produced by testicular macrophages also mediates steroidogenesis. The close physical association between the LCs and the leucocytes suggests that they are functionally related. Indeed, the

presence of ROS inhibits the mobilization of cholesterol to the mitochondria, which is a crucial step for steroidogenesis. Concurrently, the degradation of the steroidogenic machinery is also promoted by ROS and other inflammatory cytokines (139). On the other hand, sperm cells are highly susceptible to oxidative stress due to the limited amount of antioxidant machinery and cytoplasm present in mature spermatozoa (140). Obese individuals have a high metabolic rate, to restore the energetic homeostasis of the body. As a result, oxygen consumption rates increase, leading to ROS overproduction through the mitochondrial respiratory chain (141). This situation is aggravated by the permanent state of inflammation caused by the rupture of adipocytes due to triglyceride saturation (142). Consequently, macrophages invade the tissues, where pro-inflammatory cytokines are released (143). In the testicular environment, ROS is a major contributor to sperm cell dysfunction, inducing DNA damage and compromising cell membrane integrity in spermatozoa (144). Moreover, paternal obesity is associated with altered DNA methylation, meaning that methylation changes in sperm may alter the embryo development and phenotype of the offspring (145). This close relationship between inflammation and oxidative stress in the testis deserves special merit, particularly to understand the subfertility and infertility associated with overweight and obesity.

1.6. Epigenetics of obesity

Family studies involving twins and adopted children have demonstrated that adiposity is highly heritable and that the development of obesity has a strong genetic contribution (6, 146). These reports suggested that children born from obese parents could have a genetic predispose to develop metabolic disorders. Furthermore, obesity was found to be strongly related to genetics and epigenetics causes (147).

Epigenetics can be described as an ensemble of heritable changes, which can regulate gene expression without altering the DNA sequence. Genomic imprinting is an epigenetic process where genes are expressed in a similar way to the parental cell, however, to this day, the maintenance of epigenetic marks through the generation is not completely understood (148). Epigenetic marks include DNA methylation and histone modifications and many imprinted genes have been associated with growth and metabolism. When failures in imprinting occur, the expression of growth and differentiation factors is altered and metabolic disturbances, such as obesity, can be developed (147).

DNA methylation is an epigenetic regulatory mark that is found on the 5 carbon of cytosine residues. It is obtained through the addition of a methyl group to a cytosine positioned next to guanine. It actives or represses gene transcription at specific sites based on the methylations levels of the promoter region (hypermethylation leads to transcription inactivation and hypomethylation facilitates gene activation) (149, 150). Histone modifications are another type of epigenetic marks that regulate DNA transcription (151). The nucleosome is a histone octamer (a tetramer of H3 and H4 histones, flanked by two H2A-H2B dimmers) that was designed to pack DNA in a manner that allows efficient storage but, it does not allow transcriptional enzymes to access the DNA promoter. Post-translational modification of the histones can lead to promotion or repression of certain genes. For example, H3 and H4 post-translation hyperacetylation and methylation of lysine 4 and 36 of H3 histone open the DNA structure and allow gene translation. On the other way, methylation of Lysine 9, 20 27 on H3 and ubiquitination of H2A result in a closed configuration and to the repression of gene expression (151).

Recent studies have demonstrated that obese children often have obese parents (152, 153). The confirmation of this fact led to the hypothesis that early environmental influences can change epigenetic variation and therefore, affect the metabolism and increase the risk for the development of chronical diseases. Therefore, it has been a matter of great concern about how the dietary choices of the mother can have long-lasting effects on the future health of the newborns. Indeed, it is known that infants born from obese mothers are born larger for their gestational age, suggesting that infants are born with increased adiposity (154, 155).

Pregnancy is considered a natural inflammatory state due to the activation of maternal leucocytes and increased cytokines. Once combined, obesity and pregnancy induce a state of exacerbated inflammation and, consequently, promotes intra-placental inflammatory cascades and damage of placental trophoblast barrier (156, 157). Inflammation is known for inducing epigenetic modifications, especially in cancer (158). Nevertheless, little is known about how these epigenetic modifications contribute to the propagation of metabolic diseases.

Epigenetic modifications are crucial in the coordination of organogenesis and embryonic development (159). Alterations in the intrauterine environment may cause powerful epigenetic consequences. Little is known about how fetal overnutrition influences DNA methylation or other epigenetic modifications. Nonetheless, it was already reported that fetal hepatic expression of insulin-like growth factor was altered in the fetal livers of high-fat-fed mouse pups due to DNA methylation and microRNA regulation (160). Also, increased fetal lipids may favor the formation of

adipocytes over myocytes in early organogenesis (161). These studies, along with others, suggest that maternal obesity and high-fat diet consumption may lead to alterations in neurological pathways associated with appetitive, which profoundly alter offspring feeding behavior (162-164).

Regarding the paternal genetic information, it was suggested that, for the safe delivery of paternal DNA to the oocyte, it was required a replacement of canonical histones with specific sperm protamine proteins. This process of protamination would remove epigenetic modification, explaining why sperm could not transmit epigenetic changes to the embryo. Because of this, the genetic inheritance of predisposition to metabolic diseases has been attributed to the mother (165). This concept has recently been refuted. In fact, about 5-15% of the chromatin remains nucleosome-bound (165). It was postulated that retained histones may contain modifications that play an epigenetic role in embryonic regulation (166). Moreover, gene ontology analysis revealed that genes carrying histones were associated with metabolic and development processes (167). Also, the position of the histone is described as unaltered in spermatozoa from lean or obese man (167). DNA methylation may also be an important sperm epigenetic regulator. Paternal obesity is associated with altered DNA methylation, meaning that methylation changes in sperm may alter the development of the embryo and phenotype of the offspring (168).

Recent studies have suggested that male obesity may not only decrease reproductive capacity, but it can also negatively affect the offspring's health. Even though it was speculated for a long time that sperm carried potential epigenetic factors that might mediate offspring health, only in recent years this hypothesis has been taken into consideration. In mice, it was reported that males fed with a fat-diet could totally or partially transmit the obese phenotype to the offspring through subsequent generations. Later, it was verified that F0 sperm microRNA content was altered and global DNA methylation was decreased. These paramutations were inherited by the next generations (169). Another study reported that male mice fed with a high fat-diet presented increased body weight and adiposity; and impaired glucose tolerance and insulin sensitivity. The female offspring presented the same phenotype with the exception of normal adiposity, although the underlying epigenetic modifications transmitted to the offspring were not elucidated (170). One possible epigenetic factor that could induce these phenotypes could be transfer-RNA derived small RNA (tsRNA), which is a small class of non-coding RNA. A study carried out by Chen Q and colleagues revealed that sperm tsRNA from obese male mice once introduced into early healthy embryos could affect the expression of genes related to the metabolic regulation pathway. These effects were still present in the adulthood of the offspring (171).

Furthermore, significantly different sperm DNA methylation percentages were found between obese and normal-weight men at specific methylated regions (172). Genes that are involved in neurological diseases and metabolic regulation are known for been epigenetic hotspots in gametes (173). Additionally, various genes associated with appetite regulation, such as the fat mass and obesity (FTO) gene and the melanocortin-4 receptor (MC4R), were found to be methylated in a different way between sperm collected from obese and lean men (173). These results suggest that male overweight and obesity are traceable in sperm epigenome and could the transmitted to the descendants.

1.7. Monogenic obesity

According to the WHO, monogenic disorders result from a single defective gene on the autosomes, presented in all cells of the body. The mutations can be spontaneous, with no previous family history background. However, it can be inherited by future generations, usually according to Mendel's Laws. Although rare, it is hypothesized that over 10 000 human diseases have a monogenic cause (174). Even though recent genetic technology has allowed huge advances in molecular medicine, monogenic disorders are hard to study, due to its variability. Epigenetic changes, *cis* and *trans* position of the mutation are some of the variants that explain the genetic heterogeneity of these disorders. Moreover, phenotypes can be mask by other influencing factors, such as the environment and ethnicity backgrounds (175).

Monogenic obesity is caused by mutations in genes that are associated with the endocrine system. Usually, these mutations occur in the gene of the leptin/melanocortin axis, which is responsible for food intake regulation (176). Examples of single-gene disorders that also include syndromic obesity, and are associated with classic pleiotropic syndromes are the Prader-Willi (177) and Bardet-Biedl (178). These syndromes are also associated with mental retardation, dysmorphic features and organ-specific development abnormalities (176). In 1997, it was described the first human single genetic defect that leads to severe obesity without the absence of developmental delay: a mutation in prohormone convertase 1 gene causes a frameshift and a creation of a premature stop codon causing a defective prohormone processing, which leads to obesity in rodents and humans (179). In the same year, it was also reported that the deletion of a single guanine nucleotide in the leptin gene was related to severe obesity, enhancing the role of leptin as a regulator of energy balance (180). Nowadays, more than 20 single-gene disorders have been

described as autosomal forms of human obesity. These genes are classified as obesity-related genes (ORG) (181).

1.7.1. Melanocortin-4 receptor gene

The melanocortin-4 receptor is a melanocortin peptide receptor highly expressed in the central nervous system. This gene is located on chromosome 18q21.32 on the human genome and its part of a cluster of melanocortin receptors genes highly preserved during vertebrate evolution. Melanocortins are the product of pro-opiomelanocortin post-translational processing and are a member of the melanocortinergic pathways, which play an important role in the control of mammalian energy homeostasis (182). Evidences that MC4R may be an important regulator of body weight started to emerge in 1998 when it was reported for the first time that severely obese children and adults could be carriers of a mutation in the MC4R gene (183, 184). Nowadays it is known that MC4R is responsible for controlling the delicate balance between α-MSH and AgRP. The α-MSH is known for decreasing food intake in both mice and human. AgRP is an antagonist of MC4R and enhances food intake (185). Although little is known about the functional consequences of occurring mutations on the *MC4R* gene, several naturally occurring mutations in this gene have been associated with severe early-onset obesity (186). These mutations resulted in mutant receptors, which are unable to generate cAMP in response to a ligand, disrupting the melanocortinergic pathway (186). Some of the reported frameshifts and missense mutations in the *MC4R* gene did not manifest any effect in the MC4R function. However, it was also reported that 6% of the individuals with severe early-onset obesity are MC4R deficient, making this the main cause for monogenic obesity (187). These mutations are inherited in a codominant way, meaning that heterozygotes are usually less obese than their homozygous relatives (188).

A study carried out by Corrella D and colleagues revealed that Fat mass and obesity (*FTO*) gene polymorphism (specifically rs9939609) was more significantly associated with increased BMI and obesity prevalence than *MC4R* polymorphism (specifically, rs17782317). Nevertheless, both mutations had similar trends. Curiously, it was also revealed that these polymorphisms had an additive effect on the studied population, meaning that obesity prevalence was significantly higher in individuals with both mutations (189). This has been a matter of debate and remains a relevant topic for the years to come, particularly the inherited effect induced through or by MC4R. Firstly, it is essential to determine how this gene is expressed in gametes and how it relates to fertility. Nevertheless, those topics have been overlooked in the last years though there are some studies.

For instance, the expression of MC4R on single-minded homolog 1 (Sim1) neurons appears to be essential in the regulation of male sexual behavior. Mice with blocked *MC4R* expression manifest a reduced intromission efficiency and an inability to reach ejaculation. These sexual defects were reversed in mice expressing MC4R only in single-minded homolog 1 positive neurons (190). It was noticed that animals with total or partial MC4R knockout developed obesity around 12 weeks of age. Even though mice expressing MC4R only in single-minded homolog 1 positive neurons were able to mate, the authors proposed that the signaling pathways associated with mating and feeding behaviors would likely intersect through MC4R. Another study performed by the same group corroborates this hypothesis. It was reported that double-knockout mice for insulin and leptin receptors in pro-opiomelanocortin-expressing neurons manifested higher body fat percentage and subfertility in comparison to control animals. Insulin and leptin resistance induced a decrease in α-MSH production in double-knockout animals (191). Altogether, these results suggest that pro-opiomelanocortin -expressing neurons and single-minded homolog 1 neurons are part of the neural circuit underlying male sexual function where the first promotes the stimulation of the second. Although it is still not possible to explain how MC4R activity modulates sexual behavior, it was proposed that metabolic disorders, such as obesity and diabetes, could promote sexual dysfunction by impairing the MC4R signaling pathway (190, 191). Furthermore, a study performed in Holstein-Friesian bulls revealed that rs110876480, one of the most significant SNPs associated with decreased sperm motility (p=0,019), was inclosed vicinity with the *MC4R* gene (192), illustrating that there may be a close relationship between this gene and sperm quality.

1.7.2. Fat mass and obesity-associated gene

FTO gene is known to be correlated with increased BMI in both children and adults, regardless of gender. *FTO* was the first locus to be associated with adiposity, however, little is known about its specific function (193). This gene is located on chromosome 16q12.2, which are strongly conserved across various vertebrate species. Genome-wide association studies have reported that single-nucleotide polymorphisms (SNPs) associated with the intron 1 of the *FTO* gene were the most relevant for the obese phenotype(194, 195). The association of *FTO* polymorphisms and increased adiposity has been largely consistent throughout different ethnicities studies (196, 197), however, the effect that these SNPs induce on FTO structure and function is still unclear.

This gene encodes a 2- oxoglutarate-dependent nucleic acid demethylase also referred to as FTO demethylase (198). FTO has been recognized as a member of the AlkB-related family of non-haem

iron- and 2-oxoglutarate-dependent dioxygenase, which are enzymes that reverse alkylated DNA and RNA damages by oxidative demethylation (199). In addition, recent studies have examined the potential clinical implications of a dysfunctional *FTO* allele to other metabolic related dysfunctions (200-202). For example, specific *FTO* gene polymorphisms were reported to increase the risk of cancer development, such as colorectal adenomas and pancreatic cancer (203, 204). Regarding the transmission of *FTO* variants, it is suggested an autosomal-recessive mode of inheritance as it was demonstrated in a Palestinian Arab consanguineous family (205). In this family, a nonsynonymous *FTO* mutation that severely impaired the activity of FTO demethylase was equally common in both lean and obese individuals, meaning that no relationship within heterozygosity for dysfunctional *FTO* gene and obesity was found. However, all homozygous carriers for the mutation presented multiple congenital malformations and died in infancy (205). These results suggest that FTO plays a crucial role in the normal development of the central nervous system and cardiovascular system and that FTO dysfunction has the potential to induce severe polymalformation.

Regarding obesity, it was reported that 71.2 % of the patients undergoing bariatric surgery carried, at least, one risk allele for the *FTO* gene, an aspect that seems to play an important role in the long-term outcome of the surgery (206). Recently, many studies have tried to elucidate the role of *FTO* in obesity. One hypothesis involving ARID5B- FTO- IRX3/IRX5 regulatory axis was proposed by Classnitzer M and its collaborators (207). In normal adipocyte differentiation, the expression of Iroquois homeobox protein 3 (IRX3) and Iroquois homeobox protein 5 (IRX5) is repressed by AT-rich interactive domain-containing protein 5B (ARID5B), which is a repressor protein encoded by *FTO* gene. The group reported that an *FTO* variant associated with obesity, rs1421085, encoded an inactive form of ARID5B, leading to increased expression of IRX3 and IRX5. This change leads to a cell-metabolic shift from energy-dissipating thermogenesis to the increase of lipid storage (207). The link between *FTO* risk variants and IRX3/ IRX5 expression was also described in children (208).

Another hypothesis, proposed by Zhao X and colleagues, correlated the demethylation of N^6-methyladenosine (m^6A) by FTO has a possible pathway for increased adipogenesis (209). This hypothesis relies on the concept that epitranscriptomic nucleotide modifications can regulated mRNA fate, implying that mRNA carries additional information beyond the nucleotide sequence. This information is stored in nucleotide modification (such as methylations) (210). Even though the nucleotide sequence remains unaltered, translation initiation, stability, localization, and mRNA

function can be affected. The most abundant epitranscriptomic nucleotide modification is m^{6}A. In this hypothesis, m^{6}A levels are regulated by m^{6}A "erasers", enzymes able to demethylate m^{6}A (210). This epitranscriptomic factor participates in alternative splicing of mRNA during adipogenesis, since it can interact with the binding site of splicing factors, such as the serine and arginine-rich splicing factor 2. FTO was reported to have an efficient oxidative demethylation activity towards m^{6}A (211). The methylation state of m^{6}A induces alterations on the alternative splicing of several adipogenicity regulatory factors. Zhao X and colleagues demonstrated that *FTO* expression was inversely correlated with m^{6}A levels during adipogenesis. Higher levels of m^{6}A induce exon inclusion during alternative splicing (209). Although the authors could not explain how FTO and m^{6}A could induce obesity, it was highlighted that FTO could affect the alternative splicing patterns of several adipogenesis-related genes. Most importantly, it proposed that the effects of FTO on gene splicing was a possible cause for metabolic alteration, which can ultimately lead to obesity (212).

In the male reproductive tract, the expression of FTO in human testis has been identified for the first time by Landfords M and its collaborators (213). The authors identified the presence of *FTO* missense mutations in human spermatozoa from men previously diagnosed with infertility, suggesting that mutations had potential detrimental effects in the FTO RNA demethylation activity. In addition, Yang Y. and its collaborators reported that higher m^{6}A levels were present in sperm from asthenozoospermic men (low motility spermatozoa) (214). This study was based on a total of 52 semen samples, including 20 asthenozoospermic men and 32 healthy controls, in which m^{6}A modifications were accessed by liquid chromatography-electrospray ionization tandem mass spectrometry. Although *FTO* expression remained unaltered in both asthenozoospermic and normal men, the expression of alkB homolog 5 RNA demethylase (another demethylase enzyme) was decreased in the asthenozoospermic group (214). These evidences suggest that decreased sperm quality can be related to aberrant demethylation of RNA involved with male fertility. Other sperm parameters accessed by the authors did not correlate with m^{6}A levels (214).

1.7.3. Glucosamine-6- phosphate deaminase 2 gene

Glucosamine-6- phosphate deaminase 2 (GNPDA2) gene, present in chromosome 4p12 in humans, encodes the allosteric enzyme glucosamine-6-phosphate deaminase 2. This enzyme catalyzes the deamination of the D-glucosamine-6-phosphate into D-fructose-6-phosphate and its part of the hexosamine signaling pathway, one of the main nutrient-sensing pathway of the

organism (215). It seems that, under hyperglycemic conditions, it is favored the down-regulation of GNPDA2, leading to an obese and insulin-resistant phenotype, in rat fed with a high-fat diet (216). In humans, high levels of GNPDA2 expression were detected in the hypothalamus, though its role in the central nervous system has not yet been elucidated. Several genotyping studies suggested that *GNPDA2* polymorphisms are associated with obesity, in populations with different ethnic backgrounds (217-219). The same association was found in children (220, 221). Furthermore, other genome-wide works reveal that several obesity risk alleles, including *GNPDA2*, were also associated with increased insulin resistance (222, 223). Nonetheless, the clinical use of this information remains to be established. Two *GNPDA2* SNPs were also significantly associated with the development of oligozoospermia and azoospermia (224, 225). The authors aimed to identify the SNPs that could be associated with male idiopathic infertility by genome-wide association and microarray technology. The study was based on 132 Caucasian individuals from European descent, including 80 normozoospermic controls and 50 severe oligospermic or non-obstructive azoospermic individuals. Twenty SNPs were identified as significantly associated with azoospermia or oligospermia, including two SNPs associated with the *GNPDA2* gene (224, 225). However, to our knowledge, no study has investigated the impact of GNPDA2 activity in spermatozoa.

1.7.4. Transmembrane protein 18 gene

The transmembrane protein 18 (TMEM18) gene is located on the human chromosome 2p25.3, and it is part of an intergenic region, which is strongly associated with obesity (226). It is expressed in the nuclear membrane of hypothalamic brain cells and that it encodes a three-transmembrane domain protein, with a positive charge C-terminus domain. Although little is known about its function, it was hypothesized that TMEM18 can interact with DNA by its positively charged C-terminus domain, where it possibly represses DNA transcription (227). However, the structure and function of TMEM18 are still debatable. Larder R and collaborators proposed that TMEM18 is composed of four-transmembrane segments, rather than three, which are involved in the transport of proteins through the nuclear envelope, rather than transcriptional regulation (228).

Larder R and colleagues tried to elucidate the role of TMEM18 in alterations of body weight. The work performed by the authors revealed that knock-out male mice carrying a mutant TMEM18 allele have increased body weight compared with wild-type mice. Accordantly, mice in which TMEM18 was overexpressed by adeno-associated viral vectors for expression manipulation reveal

weight loss, due to an increase in energy expended (228). Interestingly, these phenotypes were not found in females, suggesting a sexually dimorphic TMEM18 expression. The sex-specific metabolic changes remain to be fully understood, yet it is very likely that gonadal hormones play an important role in metabolic dimorphisms. Dakin R and its collaborators proposed that estrogen has an important role in the control of metabolism and adiposity (229). In their experiment, both female and male mice were fed with a high-fat diet, which significantly increased males' body weight and plasma triglycerides concentration. Also, the increased serum glucose and insulin were substantially larger in male mice. Similarly, both sexes presented an increase in plasma cholesterol and hepatic triglycerides concentration. The administration of estradiol to male animals revert the increase of serum glucose and insulin, while weight and adiposity decreased. Further, the increased hepatic triglycerides were also reversed after estradiol treatment. Notwithstanding, serum testosterone concentration was diminished by estradiol treatment. With these results, the authors demonstrated that estrogen can induce metabolism and adiposity alterations, probably through alteration on the hepatic lipid metabolism (229). However, further investigation is needed to elucidate this topic with more details. Other studies have reported that TMEM18 promotor methylation and TMEM18 expression were distinct in visceral and subcutaneous adipose tissue (230, 231). Although the promoter methylation was not associated with obesity, it was proposed that TMEM18 could play an important role in the regulation of fat deposition pathways (230, 232). Landgraf F and colleagues proposed that TMEM18 is a potential regulator of the peroxisome proliferator-activated receptor gamma (PPARG) signaling, during adipogenesis. This signaling pathway participates in the regulation of several cellular events and cellular metabolism, including the peroxisomal β-oxidation pathway of fatty acids (231).

In humans, the association of SNPs near *TMEM18* gene with obesity was firstly reported by Zhao J and colleagues, which found that near-*TMEM18* SNPs induced an increase of BMI in children of European descendent (220). Since then, at least four variants of *TMEM18* SNPs have been associated with obesity (217). In adults, the results regarding the effects of *TMEM18* SNPs were inconsistent, which led to the hypothesis that *TMEM18*-associated SNPs may confer a more prominent weight gain effect in infancy and childhood than in adulthood (232). Elks and colleagues performed a genome-wide association study with 14,541 pregnant women from April 1991 to December 1992. Children's weight and height were recorded since birth until they completed 11 years of age. The authors reported that children carrying adult obesity-risk alleles, including *TMEM18*, had protection against poor weight gain during the first weeks of life. These children did

also present fast gains in height during infancy, but not in childhood. The authors assume that this fact could be explained by Karlberg model for childhood growth (233), which proposes that nutritional factors regulate growth during infancy, while endocrine factors regulate growth during childhood. Concordantly, obesity-risk alleles studied by the authors revealed a weak association with children's BMI until 3.5 years of age. However, during childhood, the authors reported that these children, although taller and heavier, now presented more fat accumulation than non-carriers of obesity-risk alleles. With these results, the authors hypothesized that adult obesity-risk alleles, including TMEM18, appear to regulate feeding behavior in the central nervous system of infants. This promotes increased growth in the early years of life, but it can also reflect the development of obesity as early as childhood (232).

1.8. The Health of future generations

In the year 2025 more than 300 million people worldwide are expected to suffer from metabolic diseases and associated complications (234). This means that more children will be born from overweight/obese parents. As we discussed, the offspring of obese individuals are at greater risk of inheriting epigenetic factors and SNPs that could carry a high risk to trigger the development of metabolic disorders later in life. In fact, it is expected an increased mortality in adult offspring of obese mothers, especially from cardiovascular diseases (235). Also, the increase in metabolic diseases is expected to be accompanied by an increase in subfertility or even infertility. More people will be seeking fertility treatments and the levels of anxiety and depression associated with involuntary childlessness are expected to rise in the years to come (236).

Prevention and treatment of obesity and other metabolic diseases is the first step to prevent health issues that will disturb modern societies in the future. Strong efforts to promote a healthier lifestyle and weight loss are starting to be made by the medical community and governments. Also, young women are starting to be sensitized about how overweight could impair their fertility and the health of their children (235). However, only recently it was suggested that male obesity could negatively affect the offspring's health. It is clear now that to protect the health of future generations is needed more than a change in the modern lifestyle. It is essential to comprehend the molecular pathways by which metabolic disorders are inherited by the offspring of overweight/obese parents and how we can prevent the genetic predisposition of individuals to develop metabolic diseases.

2. OBJECTIVES

Obesity has rapidly risen to epidemic proportions, being already classified as a major public health problem. Naturally, the number of obese individuals at reproductive age are expected to keep rising in the next decades. Since the development of obesity has a strong genetic cause, the offspring of obese individuals have higher chances of developing obesity and its comorbidities, including infertility, themselves. Unveiling of the molecular mechanisms of transgenerational inheritance of obesity will allow the identification of genetic predisposition of individuals, and better prevent the development of the disease. Although some work has been done regarding the impact of maternal obesity in offspring health, the impact of paternal obesity has been seriously disregarded and remains poorly defined.

This project aims to unveil some molecular mechanisms by which the sperm may pass information towards an overweight/obese phenotype. We hypothesized that ORG are important players in SCs metabolism and spermatozoa and that, BMI could affect the expression of these genes on sperm. Furthermore, we hypothesized that once on the spermatozoa, expression of ORG can be important for embryo development and be associated with clinical outcomes of couples seeking for Assisted-Reproduction treatments, including the pregnancy rates. For this purpose, the expression of four ORG (*MC4R, TMEM18, GNPDA2,* and *FTO)* was evaluated in human SCs and human spermatozoa, collected from testicular biopsies and human sperm samples, respectively. Therefore, the following specific objectives were established:

1. Identify the presence of ORG in SCs and human sperm;
2. Evaluate the expression of ORG in human SCs after treatment with increasing doses of obesity-associated hormones, particularly Leptin, Ghrelin, and GLP-1;
3. Evaluation of four ORG (*MC4R, TMEM18, GNPDA2,* and *FTO)* expression in human spermatozoa from normal, overweight and obese individuals;
4. Establish a correlation between the expression of the selected ORG in spermatozoa and Body Mass Index of the male individuals;
5. Establish a correlation between the expression of the selected ORG in sperm and sperm quality in males seeking for fertility treatments;

6. Establish a correlation between the expression of the selected ORG in sperm and clinical outcomes of couples seeking for fertility treatments, including embryonic development and pregnancy evolution.

Chapter II

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Human Sertoli Cell culture and experimental design

Primary human SCs were obtained from testicular biopsies collected at the Centre for Reproductive Genetics Prof. Alberto Barros (Porto, Portugal). The testicular biopsies were obtained from male patients under treatment for recovery of male gametes and used after informed written consent. All the procedures were in accordance with the Guidelines of the Local (Joint Ethics Committee CHUP/ICBAS approval number 2019/CE/P017(266/CETI/ICBAS), National and European Ethical Committee. SCs were obtained by a method optimized by Oliveira PF and colleagues. (45). In brief, testicle biopsies were washed twice in HSSBf (Hanks Balanced Salt Solution without Ca^{2+} and Mg^{2+}) and centrifuged at 300 g for 5 min. The resulting pellet was suspended in SCs culture medium (DMEM: Ham's F12, 1:1, containing 15 mM HEPES, 50 U/mL penicillin, and 50 mg/mL streptomycin sulfate, 0.5 mg/mL fungizone, 50 μg/mL gentamicin and 10% heat-inactivated FBS). All chemicals and the medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Large cell clusters were disaggregated by forcing the pallet through a 20G needle. The cells were then plated in cell culture flasks and incubated at 37° C, 6% CO_2. After 96 hours, the cultures presenting cell contaminants over 5% were discarded. To test the purity of the culture it was used an ExtrAvidin Peroxidase Staining Kit (Sigma-Aldrich, St. Louis, MO, USA).

3.1.1. Experimental Design

SCs were cultured in the presence of leptin, ghrelin and, GLP-1 (purchased from Sigma-Aldrich, St. Louis, MO, USA). To establish the various experimental groups, SCs were firstly washed with phosphate-buffed saline (PBS) and plated in a serum-free medium (DMEM: Ham's F12, pH 7.4) supplemented with insulin-transferrin-sodium selenite medium (final concentration of 10 mg/L; 5.5 mg/L; 6.7 μg/L, respectively). To each obesity-related hormone, three different experimental conditions were tested. A control group, without the addition of any obesity-related hormone, was done. For the leptin experiment, SCs were exposed to (in ng/mL): 5, 25 and 50. The concentration of 5 ng/mL complies with the physiological levels of leptin reported for lean, healthy men. The concentration of 25 ng/mL corresponds to the physiological levels of leptin reported for obese men. Finally, the concentration of 50 ng/mL was chosen to mimic the leptin concentrations reported in morbidly obese men. For ghrelin experiment, SCs were exposed to (in pM): 20, 100

and 500. These concentrations were chosen based on the physiological levels of ghrelin reported for obese, normal, and severe malnourish individuals, respectively. For GLP-1 experiment, SCs were exposed to (in pM): 10, 1000 and 1×10^5. The concentration of 10 pM complies with the physiological levels of GLP-1 reported to healthy individuals. The other two concentrations were chosen based on the plasmatic concentration of GLP-1 found in individuals after the administration of a GLP-1 analog in obesity treatment therapies. The concentration of 1000 pM was based on the plasmatic concentration of GLP-1 found in obese individuals after a single dose (3 mg per day). The concentration of 1×10^5 pM was chosen to represent the plasmatic concentration of GLP-1 found in obese individuals after daily administration for 5 weeks.

SCs were treated for 24 hours with leptin and ghrelin and for 6 hours with GLP-1. After treatment, the cell culture medium was collected, and the cells detached from the culture flask with a trypsin-EDTA solution. Trypan Blue Exclusion test was used to evaluate cell viability (averaging 85-90%). After counting the cells with a Neubauer chamber, cells were collected for RNA extraction.

3.2. Patient's Characterization and Study Design for Spermatozoa

Human sperm samples were collected at the Centre for Reproductive Genetics Prof. Alberto Barros (Porto, Portugal). This study was based on a cohort of 106 infertile couples undergoing assisted reproduction treatments on the Centre during the period of October 2018 to January 2019. The population included 17 *in vitro* fertilization (IVF) cycles and 89 intracytoplasmic sperm injections (ICSI) cycles. Patients who had suffered from an inflammatory disease prior to sample collection were discarded. Also, patients who had suffered from cancer or other diseases that could influence fertility were dismissed. An exception was made for patients suffering from metabolic diseases. Patients were asked to disclose their tobacco habits and the consumption of other substances that could interfere with the infertility treatment. Men's average age was 39.5 years old. Semen samples were collected by masturbation into sterile containers, after a period of 2-4 days of abstinence. To the 15 patients diagnosed with obstructive azoospermia (A), sperm cells were collected by epididymal aspiration, a relatively straightforward technique for spermatozoa recovery. Semen analysis was performed according to the World Health Organization guidelines (237), along with the Local, National and European Ethical Committee guidelines. The samples were washed and submitted to seminal processing by swim-up technique (238). This technique is based on the movement of pre-washed spermatozoa in an overlying medium. The fraction containing the motile normal spermatozoa which, actively migrated from the pallet to the upper aqueous phase, is the

one used for performing the assisted-reproductive techniques. Sperm parameters were evaluated by certified embryologists. What was left from this fraction after the performance of the assisted-reproductive techniques was used for the ORG genes expression studies. Once in our laboratory, the samples were centrifuged at 8000 *g* for 5 min, at 4° C. The resulting pellet was washed with PBS solution. Finally, the samples were centrifuged at 16 000 *g* for 10 min, at 4° C. The supernatant was discarded, and the pellets preserved at -80° C until they were used for RNA extraction.

3.2.1. Body Mass index determination

Male patients were characterized by their body mass index (BMI), a simplified measure of the body fat index based on the height and weight of an adult person. Paternal weight and height were collected by the clinical staff. Male patients were then grouped according to BMI as follows: normal weight (<25 kg m^{-2}), overweight (25-29.9 kg m^{-2}) and obese (>30 kg m^{-2}).

$$\text{BMI}\left(\frac{\text{kg}}{\text{m}^2}\right) = \frac{\text{Weight (kg)}}{[\text{Height (m)}]^2}$$

The BMI ranges used in this study were based on the standard nutritional status for adult individuals reported by the WHO (239).

3.2.2. Assisted-Reproduction Techniques data analysis

Sperm quality parameters and data regarding embryo development and pregnancy evolution were collected at the Centre for Reproductive Genetics Prof. Alberto Barros. Embryo development was evaluated through embryo quality parameters, which include fertilization rate, embryo cleavage rate, high-quality embryo rate, and blastocyst rate. Embryo quality parameters were calculated by the following formulas:

$$\text{Fertilization Rate} = \frac{\text{Nº } 2 - \text{pronuclear embryos}}{\text{Nº Mature Oocytes (MII)}}$$

$$\text{Embryo Cleavage Rate} = \frac{\text{Nº } 4 - \text{cell stage embryos}}{\text{Nº Mature Oocytes (MII)}}$$

$$\text{High} - \text{quality Embryo Rate} = \frac{\text{Nº High} - \text{quality } 8 - \text{cell stage embryos}}{\text{Nº Mature Oocytes (MII)}}$$

$$\text{Blastocyst Rate} = \frac{\text{Nº blastocyst stage embryos}}{\text{Nº Mature Oocytes (MII)}}$$

Embryo development after embryo transference was evaluated by the biochemical pregnancy rate (BP), which was calculated by the following formula. A statistical analysis of the number of Ongoing pregnancies and Abortions was also performed.

$$\text{BP} = \frac{[\text{Serum } \beta - \text{human chorionic gonadotropin}]\ (\text{mIU.mL}^{-1})}{\text{Embryo Transfer Cycles}}$$

3.3. RNA isolation from human Sertoli cells and human sperm

SCs total RNA extraction was performed using the Total RNA kit (Jena Bioscience, Jena, Germany) as indicated by the manufacturer. Briefly, to pelleted cells (around 1 to 5×10^6 cells) was added a lysis buffer (accordingly to cell concentrations) and, after repetitive pipetting, samples were vortexed for 10 s. Next, it was added 300 µL of isopropanol to the lysates. The mix was immediately transferred to a column provided by the kit. After washing the column with the washing buffers provided, the RNA was eluted from the column with an elution buffer (40 µL).

Human sperm total RNA extraction was performed using the RNeasy Mini kit (Qiagen, Hilden, Germany) as indicated by the manufacturer. Briefly, to sperm pellets (100 to 5×10^6 cells) was added 350 µL lysis buffer (RLT buffer) and after repetitive pipetting, samples were vortexed for 30 s. Next, it was added 350 µL of ethanol 70% to the prepared lysates. The mix was immediately transferred to a column provided by the kit. After washing the column with the washing buffers provided (RW1 buffer, and RPE Buffer), the RNA was eluted from the column with RNase-free water (30 µL).

RNA concentrations and absorbance ratios (A_{260}/A_{280} and A_{260}/A_{230}) were determined by NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). The RNA samples were stored at -80° C until use.

3.4. Complementary DNA (cDNA) synthesis

Total RNA was reverse transcribed in a final volume of 20 µL by M-MuLV Reverse Transcriptase (NZYTech, Lisbon, Portugal) according to manufacturer instructions. Briefly, 2.5 µL of Random hexamer primers (50 ng/µL) (NZYTech, Lisbon, Portugal) and 1 µL of deoxynucleotides (dNTPs) (NZYTech, Lisbon, Portugal) was added to each sample, containing 0.07 µg of RNA. The volume

was adjusted to 17 µL with RNase-free water. For RNA denaturation, samples were incubated at 65° C for 5 min. After adding M-MuLV Reverse Transcriptase and the respective enzyme buffer to each sample, the samples were incubated concordantly to the manufacture protocol: 10 min at 25° C, for reagent activation, 50 min at 37° C, for conversion, and 15 min at 70° C for enzyme inactivation.

3.5. Polymerase chain reaction (PCR)

To identify the ORG transcripts in SCs and sperm we performed PCR assays according to standard methods. In brief, exon-exon spanning primers were designed for the human genes: *MC4R, GNPDA2*, *TMEM18,* and *FTO*. To amplify the desired genes, it was used NZYTaq II 2x Green Master Mix (NZYTech, Lisbon, Portugal), which is a premixed ready to use a solution containing a Taq-derived DNA polymerase, dNTPs, reaction buffer and other additives to optimize the reaction. cDNA was amplified in a final volume of 12.5 µL. To each sample, it was added 1 µL cDNA (1 µg/µL), 0.1 µL of primer forward (50 µM), 0.1 µL of primer reverse (50 µM) and 6.5 µL of Master Mix. The volume was adjusted to 12.5 µL with RNase-free water. The conditions of PCR cycles were adjusted to better fit optimal temperatures of enzyme activation, DNA denaturation, annealing, and dissociation. The protocol for PCR was the following: 5 min at 95° C for enzyme activation, 30 s at 95° C for DNA denaturation, 1 min at 72° C for extension and, 7 min at 72° C for final denaturation. The annealing temperatures were adjusted to each pair of primers and can be consulted in **Table 2.** The samples were run in a desaturating agarose gel (3 %), stained with GreenSafe (NZYTech, Lisbon, Portugal). The results were revealed in the ChemiDoc™ XRS+ System (Bio-Rad, California, USA).

3.6. Real-Time Polymerase chain reaction (qPCR)

To perform the qPCR assays, we used qPCR Green Master Mix (2x) (NZYTech, Lisbon, Portugal) which, already contains a DNA polymerase enzyme, buffer and SYRBR green nucleic acid stain. cDNA was amplified in a final volume of 16 µL. To each sample, it was added 0.8 µL of cDNA, 0.64 µL of primer forward (10 µM), 0.64 µL of primer reverse (10 µM) and, 8.0 µL of Master Mix. The volume was adjusted to 16 µL with RNase-free water. The conditions of qPCR cycles were adjusted to better fit optimal temperatures of enzyme activation, DNA denaturation, annealing, and dissociation. The protocol for qPCR was the following: 10 min at 95° C for polymerase activation, 15 s at 95° C for DNA denaturation and, 1 min at 72° C for extension. The annealing temperatures

and the number of cycles required for the exponential phase of amplification were optimized to each set of primers. Along with the primer sequences, the annealing temperatures and the number of cycles can be consulted in **Table 2**. Melting curve was determined by an additional step: 1 min at 95° C, 30 s at 55° C and 30 s at 95° C. qPCR reactions were carried in duplicate and the optical density was assessed by a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, California, USA).

Table 2: Primer sequences and PCR conditions used to assess gene expression and mRNA abundance in human SCs and spermatozoa. *GAPDH* and *β2-microglobulin* were used as housekeeping controls.

Gene	GenBank	Primer sequence (5'-3')	Annealing Temperature	Amplicon Length	Cycles
hMC4R	NM_005912.2	Sense: GACAGTTAAGCGGGTTGGGA Anti-sense: GACGCCAATCAGGATGGTCA	58° C	277 bp	35
hGNPDA2	NM_138335.2	Sense: TGTTCCAGCGTCGAAGAACC Anti-sense: CTGCCCATTCACTAGCCAAG	58° C	117 bp	35
hGNPDA2	NM_138335.2	Sense: GGTTCCTTCCTGCGCCTTTA Anti-sense: ACAAGTTCAAACCGGCAGAC	64° C	149 bp	35
hTMEM18	NM_001352680.1	Sense: TCGGGCACTTTCTGTGTCTAGT Anti-sense: GAAGGACGCAAACTCCAAGCAG	58° C	298 bp	35
hFTO	NM_001080432.2	Sense: GAGCGCGAAGCTAAGAAACTG Anti-sense: ATACACTGCTGGCTTCTCGG	59° C	142 bp	35
hGAPDH	NM_002046.7	Sense: CGCCAGCCGAGCCACATC Anti-sense: CGCCCAATACGACCAAATCCG	58° C	93 bp	35
hβ2-MGB	NM_004048.3	Sense: AGATGAGTATGCCTGCCGTG Anti-sense: TCATCCAATCCAAATGCGGC	58° C	119 bp	35

The expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β2-microglobulin (*β2-MGB*) was used as internal control for the expression of *MC4R, GNPDA2, TMEM18*, and *FTO*.

3.7. Western-Blot (WB)

The pellet of SCs and human sperm was collected, washed with PBS and dried by centrifugation (8000 *g*, 10 min at 4º C). The pellets were then diluted in 50 µL of RIPAS Buffer (1% Noridet P-40, 0.5% Sodium deoxycholate, 0.1% Sodium dodecyl sulphate 10% in PBS, 0.1% Phenylmethanesulfonyl fluoride 100 mM, 0.1% cocktail mix of protease and phosphatase inhibitors, 0.1% sodium orthovanadate 100 mM) and homogenized with a microtubule piston. The samples were let to rest for 20 min at 4ºC and then centrifuged at 14 000 *g,* 4º C, 20 min. The pellet of cellular debris was discarded, and protein concentration of the supernatant was quantified by Pierce Bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Massachusetts, USA).

Proteins samples (50 µg) from SCs and human sperm were prepared with electrophoresis sample buffer (0.125M Tris-HCl pH 6.8; 4% SDS; 20% glycerol, 10% 2-mercaptoethanol), fractionated on an SDS-PAGE system with TGX™ Stain-Free Acrylamide gel (Bio-Rad, Hercules, California, EUA), 30 mA per gel for 90 minutes, at room temperature, and, transferred to polyvinylidene difluoride membranes (100 V for 60 minutes, at 4° C). The membranes were then blocked in a Tris-buffered saline solution (TBS) with 0.05% Tween 20 containing 5% non-fat dry milk. Before incubation with the primary antibody, the membrane was washed 3 times with washing buffer (TBS with 0.05% Tween 20), 5 minutes each time. The membrane was incubated with a primary antibody, diluted in TBS with 0.05% Tween 20 with 1% non-fat dry milk, overnight, at 4° C. The list of antibodies used in this project, along with the concentrations used, can be consulted in **Table 3**. Afterward, the membranes were washed with the washing buffer and exposed to the appropriate peroxidase-conjugated secondary antibody for 1h and 30min, at room temperature. The secondary antibody was diluted in TBS with 0.05% Tween 20 with 1% non-fat dry milk (1:5000). After washing, the membranes were exposed to a chemiluminescence detection reagent and analyzed by ChemiDoc^{Tm} MP Imaging system (Bio-Rad, California, USA). Total protein was accessed by fluorescence for loading control.

3.8. Immunofluorescence staining (IF)

Human SCs were plated ($10x10^3$ cells/well) in 12-well culture plates with coverslips and incubated at 37º C in 6% CO_2 humidified atmosphere until a 60% cell confluence is achieved. Before the fixation step, the coverslips were washed 3 times with PBS. To each coverslip, it was added 200 µL of 10% neutral formalin. After, incubation at room temperature for 20 min, coverslips were washed and kept in PBS, at 4º C, until the next step.

To fix the spermatozoa to the coverslips, a pellet of fresh spermatozoa was dissolved in 10% neutral formalin and left to incubate at room temperature for 20 min. A sample volume corresponding to 50×10^3 cells was transferred to each coverslip. With the help of the pipette tip, the sample was spread throughout the entire area of the coverslip. To dry the samples, each slide (containing two coverslips) was incubated at 37ºC, 20 min. Afterward, coverslips were washed and kept in PBS, at 4º C, until the next step.

For cell membrane permeabilization, the coverslips were incubated, at room temperature, with 0.1% Triton 100 × PBS, for 15 min. After further washing with PBS (3 times), the coverslips were blocked 0.1% gelatin PBS (15 min) and incubated with a primary antibody, diluted in 0.1% gelatin PBS, overnight at 4º C. The list of antibodies used in this project, along with the concentrations used, can be consulted in the **Table 3.**

The coverslips were washed with 0,1% gelatin PBS (3 times) before their incubation with a correspondent Alexa-fluor secondary antibody, for 1h, at room temperature. The coverslips were rinsed with 0.1% gelatin PBS (3 times). In a microscope slide was mounted with a drop of VectaShield (Vector Laboratories, Burlingame, USA) and the coverslips (cells facing the VectaShield). The microscope slides were visualized in a Nikon Eclipse E400 microscope equipped with a Y-FL Epi-Fluorescence Attachment and HB-10103AF Super High-Pressure Mercury Lamp Power Supply (Nikon, Shinagawa, Tokyo, Japan), coupled with Nikon NIS Elements Image Software.

Table 3: List of antibodies, and respective concentrations, used for identification of proteins associated with ORG expression.

Antibody	Company	Catalog Number	Concentration for WB	Concentration for IF
Anti-FTO	Abcam	ab92821	-	1:50
Anti-FTO	Abcam	ab94482	1:500	-
Anti-MC4R	Abcam	ab24233	1:500	1:50
Anti-TMEM18	Abcam	ab100954	1:1000	1:50
Anti-GNPDA2	Abcam	ab106363	1:1000	1:50
Goat Anti-Mouse IgG-HRP conjugated	Sigma Aldrich	AP308P	1:1500	-
Mouse Anti-Rabbit IgG-HRP conjugated	Santa Cruz Biotechnology	sc-2357	1:1500	-
Goat Anti-Mouse IgG-Alexa Fluor 546 conjugated	Thermo Fisher Scientific	A-11003	-	1:1500
Goat Anti-Rabbit IgG-Alexa Fluor 488 conjugated	Thermo Fisher Scientific	A-11008	-	1:1500

3.9. Statistical Analysis

All data presented are expressed as mean ± SEM. The association between ORG mRNA expression in sperm, male BMI, male age and other clinical parameters were evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution and a confidence interval of 95%. Values of $P<0.05$ were considered as statistically significant. Statistical analysis of the association between ORG mRNA abundance and appetite-regulating hormones treatment was performed using analysis of variance (ANOVA) followed by Tukey post-hoc test for multiple comparisons. Values of $P<0.05$ were considered as statistically significant. The statistical analysis of this work was performed using GraphPad Prism 6 (GraphPad Software Inc., SanDiego, CA, USA).

Chapter III

RESULTS AND DISCUSSION

4. RESULTS

4.1. Identification of ORG in human Sertoli cells and human Spermatozoa

ORG and epigenetic factors have been proved to be involved in the development of overweight/obese phenotype, particularly because children born from obese parents have been suggested to have a genetic predisposition to develop metabolic disorders themselves. Since metabolic disorders, such as obesity, are known to alter SCs metabolism and thus the nutritional support of spermatogenesis, affecting sperm quality, we hypothesized that ORG could be part of the mechanism by which obesity promotes a decreased fertility potential on men. Our first task was to identify the presence of ORG transcripts in both human SCs and human spermatozoa (Figure 5).

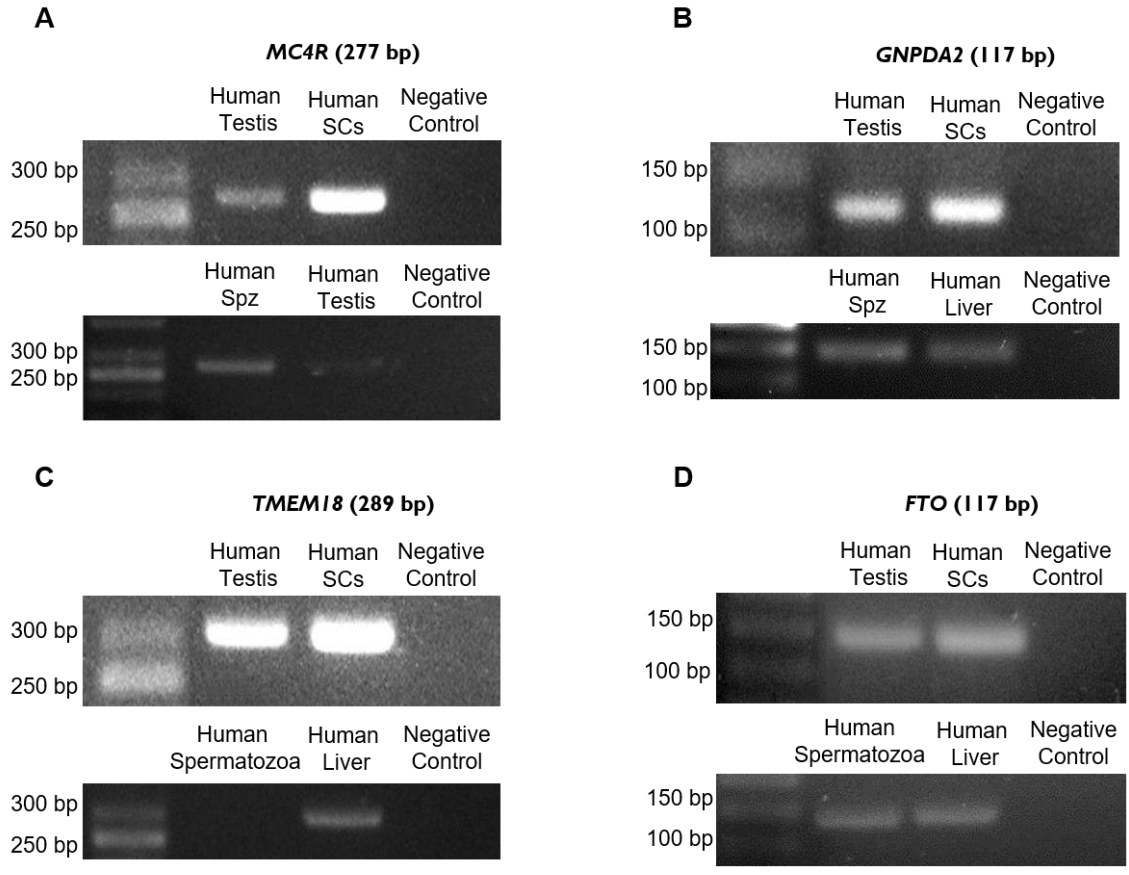

Figure 5: Identification of ORG in human Sertoli cells and spermatozoa by conventional PCR. Conventional PCR was used to identify the presence of *MC4R* (A), *GNPDA2* (B), *TMEM18* (C) and *FTO* (D) transcripts in both human SCs and spermatozoa. A cDNA-free sample was used as a negative control. Human testis and human liver samples were used as positive controls.

By conventional PCR, we investigated the presence of *MC4R, GNPDA2, TMEM18* and *FTO* transcripts in both SCs and spermatozoa. We identified, for the first time, the presence of *MC4R* and *GNPDA2* transcripts in both human SCs and spermatozoa (**Figure 5, panel A and B)**. Although the presence of *TMEM18* mRNA was not identified in human spermatozoa, it was identified, for the first time, in human SCs (**Figure 5, panel C)**. Additionally, we further confirmed the presence of *FTO* mRNA in human SCs and spermatozoa, has it had been already reported by Landfors and colleagues (213) (**Figure 5, panel D)**. The presence of ORG proteins on human SCs and spermatozoa was further investigated by WB and IF. The GNPDA2 and TMEM18 proteins were detected in both SCs and spermatozoa by WB **(Figure 6, panel B and C)**. The presence of MC4R was only detected in human SCs (**Figure 6, panel A**), while the presence of FTO was not detected in either cell (**Figure 6, panel D**).

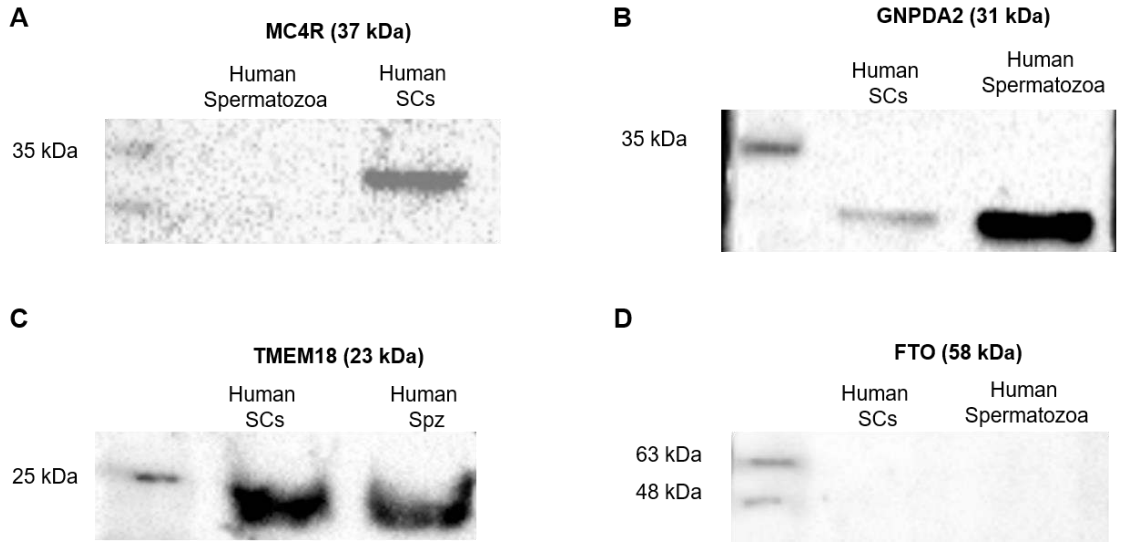

Figure 6: Identification of ORG proteins in human Sertoli cells and spermatozoa by Western-Blot. (A) represents the identification of MC4R in human SCs. The presence of this protein in human spermatozoa was not detected by WB. Both GNPDA2 (B) and TMEM18 (C) were present in both human SCs and spermatozoa. However, FTO (D) was not present in human SCs nor in spermatozoa.

The location of ORG proteins in human SCs and human spermatozoa was accessed by IF (**Figure 7**).

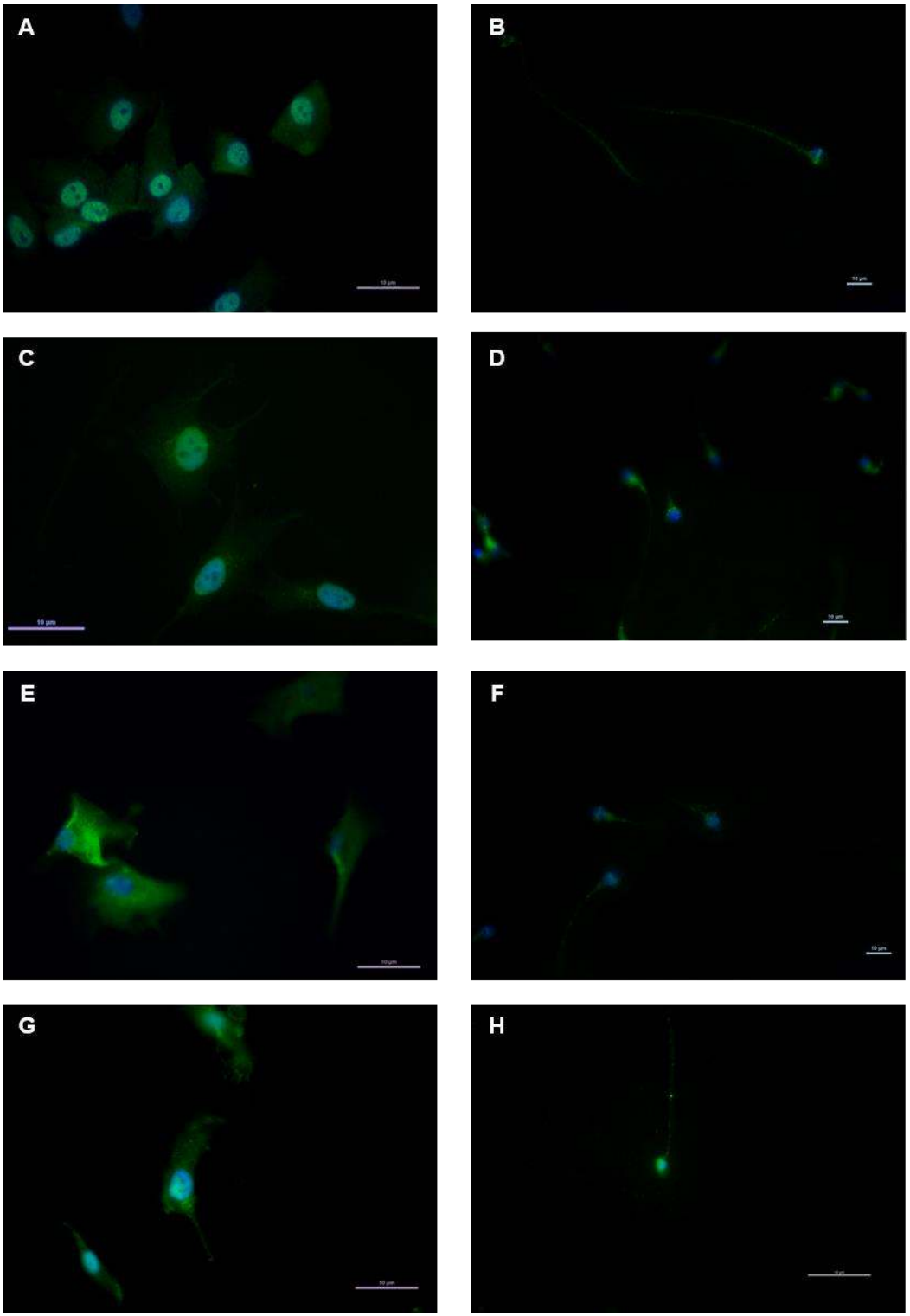

Figure 7: The results of immunofluorescence staining for the studied ORG in human Sertoli Cells and Spermatozoa. All images represent the merge results between the DAPI channel and the Alexa Flour 546/488 channel. MC4R was detected in SCs (A) and spermatozoa (B). GNPDA2 was detected in SCs (C) and spermatozoa (D). TMEM18 was detected in SCs (E) and spermatozoa (F). FTO was detected in SCs (G) and spermatozoa (H). Cells incubated only with Alexa Fluor secondary

antibody were used as negative control and the corresponding images can be found in the supplementary section of this report.

By IF, we could identify the presence of all ORG proteins studied in both human SCs and spermatozoa. MC4R was present in both SCs and spermatozoa (**Figure 7, panel A and B**).
On SCs, MC4R was highly expressed in the nuclear envelop and cytoplasm (**Figure 7, panel A**), while it was majorly expressed in the cytoplasmatic membrane of spermatozoa (**Figure 7, panel B**). Similarly, GNPDA2 was also highly expressed in the nuclear envelope of SCs (**Figure 7, panel C**). On spermatozoa, GNPDA2 appeared to be more expressed in the cytoplasm and mainly on spermatozoa's head region (**Figure 7, panel D**). TMEM18 was highly expressed in the cytoplasm of both SCs (**Figure 7, panel E**) and spermatozoa (**Figure 7, panel F**). Similarly to GNPDA2, TMEM18 appears to be present mainly on spermatozoa's head region. FTO was also present in the cytoplasm of both SCs (**Figure 7, panel G**) and spermatozoa (**Figure 7, panel H**). Along with spermatozoa's head region, the presence of FTO on spermatozoa's midpiece and the tail is also clear. With these results, we identify, for the first time, the presence of GNPDA2, MC4R, TMEM18, and FTO proteins in human spermatozoa. We also identify, for the first time, the presence of MC4R, GNPDA2, and TMEM18 in human Sertoli cells. We further confirm that FTO is present in human SCs as previously reported (213).

4.2. Expression of ORG in human Sertoli Cells after stimulation with obesity-related hormones

The hormonal dysregulation associated with obesity is responsible for the disruption of several reproductive events. Previous studies showed that leptin-ghrelin axis and GLP-1, which mediate body energy homeostasis, interfere with SCs function and metabolism (28, 65, 71) and may induce implications in the nutritional support of spermatogenesis. We hypothesize that beyond the already known metabolic effects induced by the hormonal dysregulation caused by overweight/obesity, the expression of ORG in SCs also responds to leptin, ghrelin, and GLP-1 fluctuations.

4.2.1. GNPDA2 expression is increased in human SCs after treatment with leptin concentration found in obese men

Leptin is a major regulator of appetite. Obese individuals are known to suffer from leptin resistance which is characterized by high levels of circulating leptin in the bloodstream. High levels of leptin are known to interfere with the nutritional support of spermatogenesis, promoting the decrease production of acetate and lactate by SCs (28), both essential for developing germ cells. Subsequently, we hypothesized that ORG expression by SCs could also respond to the leptin stimuli. We treated human SCs from testicular biopsies with increasing doses of leptin, 5 ng/mL, 25 ng/mL and 50 ng/mL, corresponding to the serum concentration levels of this hormone in normal, overweight and obese individuals (**Figure 8**).

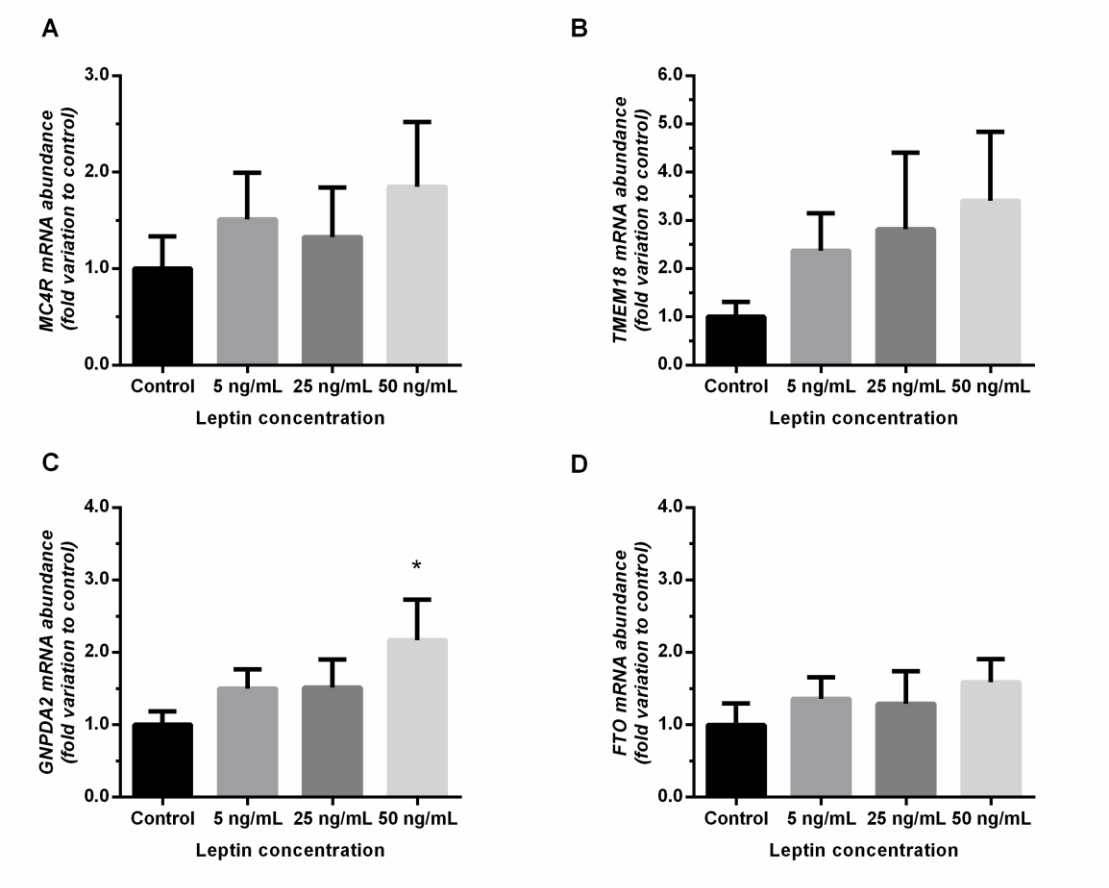

Figure 8: Effect of leptin on the expression of ORG in human Sertoli Cells. The expression of ORG (A: MC4R; B: TMEM18; C: GNPDA2 and D: FTO) by human SCs, after exposure to increasing doses of leptin (24h of treatment) was accessed by quantitative polymerase chain reaction. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($p < 0.05$) are as indicated: * relative to control.

We could not detect any alteration on the expression of *MC4R, TMEM18,* and *FTO* on SCs after stimulation with leptin (**Figure 8, panels A, B, and D,** respectively). However, stimulation of human SCs with 50 ng/mL of leptin, which mimics the serum levels of this hormone found in morbidly obese individuals, increased *GNPDA2* expression (**Figure 8, panel C**). This result can be related to the role that GNPDA2 performs on the hexosamine signaling pathway and, its deamination action towards D-glucosamine-6-phosphate, a topic that will be further discussed.

4.2.2. TMEM18 expression is increased in human SCs after treatment with ghrelin concentration found in normal men

While overweight/obesity is associated with the rise of serum leptin levels, the opposite occurs with ghrelin. As discussed before, ghrelin is known to promote food intake and insulin secretion, which means that under hyperglycemic conditions, such as in obesity or diabetes, the secretion of this hormone is down-regulated. A previous study from our group revealed that glucose consumption and mitochondrial membrane potential of SCs are sensitive to ghrelin levels, suggesting that ghrelin acts as an energy sensor for human SCs, in a dose-dependent manner (65). After this, we hypothesized that expression of ORG by SCs could also respond to the different concentrations of ghrelin. To test our hypothesis, we treated human SCs with increasing doses of ghrelin, 20 pM, 100 pM, and 500 pM, corresponding to the serum concentration levels of this hormone reported in obese, normal and undernourished individuals (**Figure 9**). We could not detect any alteration on the expression of *MC4R, GNPDA2,* and *FTO* on SCs after stimulation with ghrelin (**Figure 9, panel A, C, and D**, respectively). Interestingly, the expression of *TMEM18* was increased after SCs stimulation with 100 pM of ghrelin which mimics the serum levels found in normal-weight individuals (**Figure 9, panel B**). These results suggested that, in SCs, *TMEM18* expression is decreased upon abnormal metabolic situations, such as obese and/or underweight. However, the role that *TMEM18* can play on the metabolic regulation of SCs is still unknown.

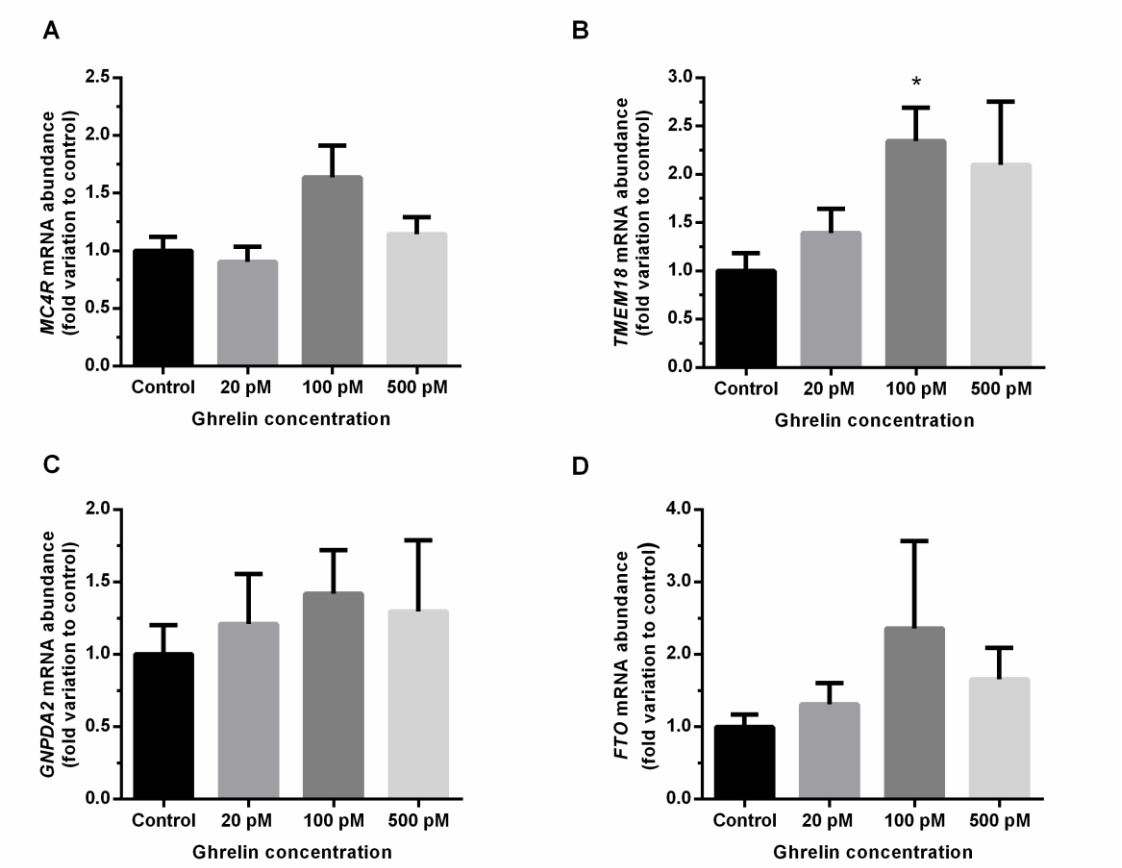

Figure 9: Effect of ghrelin on the expression of ORG in human Sertoli Cells. The expression of ORG (A: *MC4R*; B: *TMEM18*; C: *GNPDA2* and D: *FTO*) by human SCs after exposer to increasing doses of ghrelin (24 hours of treatment), was accessed by quantitative polymerase chain reaction. Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($p < 0.05$) are as indicated: * relative to control.

4.2.3. Treatment of human SCs with different GLP-1 concentrations has no effect on the expression of the selected ORG

Along with leptin and ghrelin, GLP-1 is an appetite- associated hormone that can change glucose homeostasis being capable of promoting satiety and decreased caloric intake while promoting the secretion of insulin, proliferation of islet, coupled with inhibition of glucagon secretion. Its physiological action promoted the use of GLP-1 analogs for the treatment of diabetes, a condition known by hyperglycemic events and insulin resistance (69). Furthermore, GLP-1 levels appear to be associated with obesity even though, in those conditions, GLP-1 levels are inconsistent and its variations have not been conclusively determined (119, 120). Additionality, previous work from our group showed that GLP-1 is able to modulate glucose consumption and lactate production by SCs,

suggesting that GLP-1 is also a modulator of the nutritional support of spermatogenesis (71). We hypothesized that GLP-1 administration on hyperglycemic obese individuals could also modulate the expression of ORG on SCs. To test that hypothesis we treated human SCs with increasing concentrations of GLP-1, 10 pM, 1000 pM, and 1×10^5 pM, corresponding to the serum concentration levels of this hormone in normal individuals, obese individuals after a single dose of GLP-1 analogue (3 mg) and obese individuals after daily administration (3 mg) for 5 weeks (**Figure 10**).

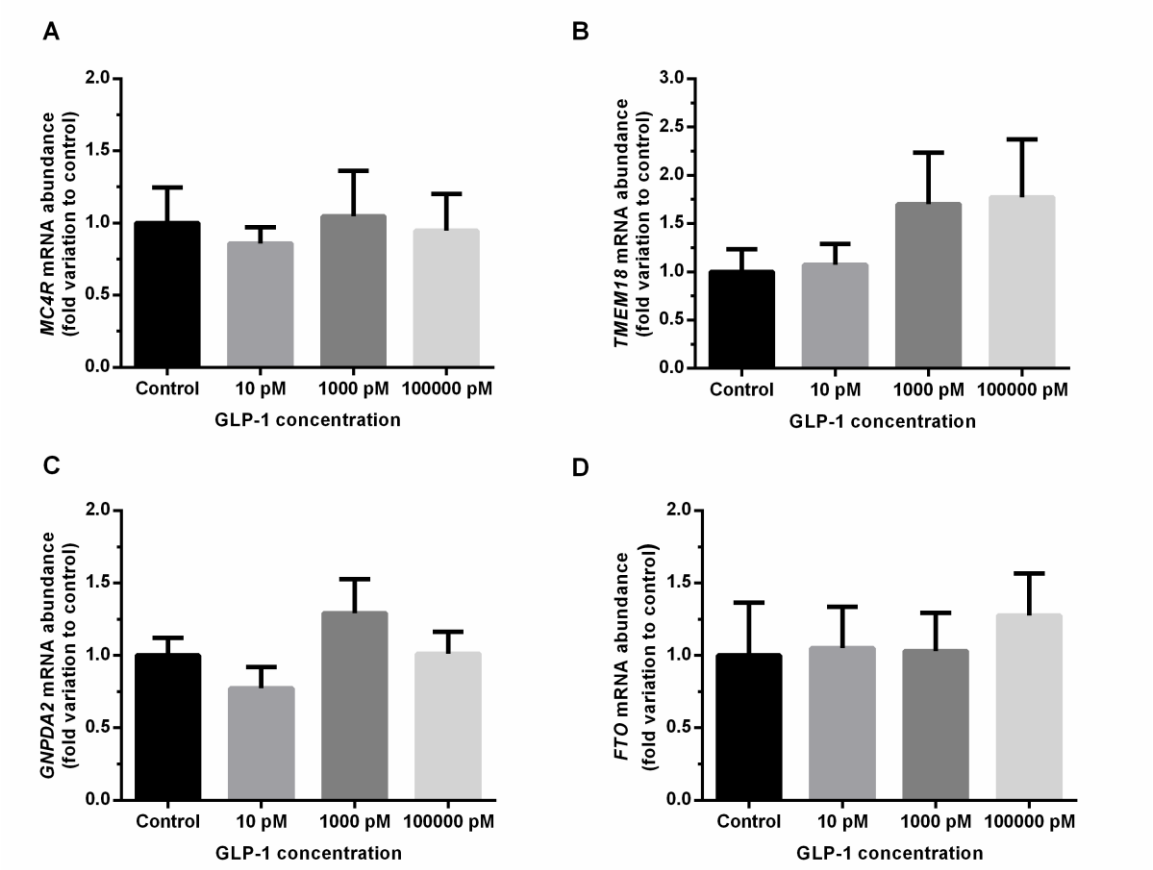

Figure 10: Effect of GLP-1 on the expression of ORG in human Sertoli Cells. The expression of ORG (A: *MC4R*; B: *TMEM18*; C: *GNPDA2* and D: *FTO*) by human SCs, after exposer to increasing doses of GLP-1 (6 hours of treatment), was accessed by quantitative polymerase chain reaction. Results are expressed as mean ± SEM (n=6 for each condition). No results between the studied parameters were found to be statistically significant.

Interestingly, we could not detect any alteration on the expression of *MC4R, TMEM18, GNPDA2*, and *FTO* on human SCs, after treatment with GLP-1 (**Figure 10, panels A, B, C, and D**, respectively). These results lead us to confirm that the administration of GLP-1 analogs to hyperglycemic obese

individuals did not alter the expression of ORG by SCs. In addition, our data suggest that effects on the expression of ORG mediated by obesity-related hormones are not modulated by GLP-1.

4.3. TMEM18 is not expressed in human spermatozoa and there is no correlation between the expression of ORG by human spermatozoa and body mass index

The previous results suggested that the expression of ORG by human SCs is modulated by obesity-related hormones, specifically leptin and ghrelin. Furthermore, it was showed that hormonal dysregulation associated with obesity alters the expression levels of ORG genes in SCs. As it was previously mentioned, SCs are the sentinels of spermatogenesis, meaning that every stimulus that reaches the developing germ cells as to first overcome the BTB barrier. Considering that our results suggest that the hormonal dysfunction associated with obesity alters the expression of ORG in human SCs, we hypothesized that overweight/obesity could also influence the expression levels of ORG on human spermatozoa. To test our hypothesis, we evaluated *MC4R, GNPDA2* and *FTO* mRNA abundance of 106 sperm samples (average male age of 39.5 years old) by qPCR and compare it with the BMI of the male individuals (average BMI of 26,5 kg/m^2). The presence of *TMEM18* transcript was not present on human spermatozoa (**Figure 5, panel C**). We could not detect any correlation between the abundance of the *MC4R, GNPDA2* and *FTO* transcripts in spermatozoa and BMI (**Figure 11, panels A, B, and C,** respectively), suggesting that the expression of ORG, in spermatozoa, is not directly affected by paternal BMI.

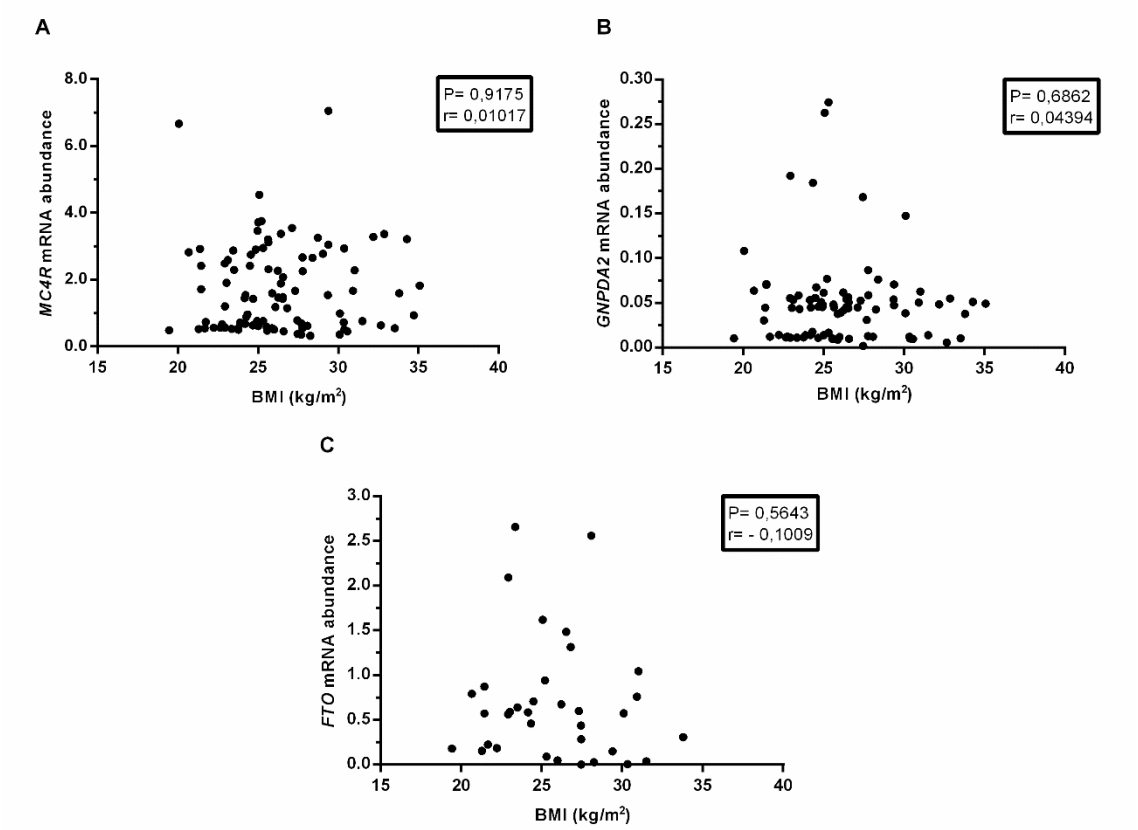

Figure 11: Pearson correlation between ORG expression in human spermatozoa and paternal body mass index (BMI). The association between BMI (average of 26,5 kg/m²) and (A) *MC4R* (n=95), (B) *GNPDA2* (n=87), and (C) *FTO* (n=35) mRNA abundance was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). No correlations between the studied parameters were found to be statistically significant.

4.4. There is no correlation between the expression of ORG in human spermatozoa and paternal age

Senescence is known to induce several metabolic changes in the testis. Among the several physiological alterations associated with aging, hormonal dysregulation has been pointed out as one of the main causes of the decrease in the fertility potential of older men. Hormonal dysregulation associated with senescence is primarily caused by low testosterone levels, a condition also known as hypogonadism (240). Hypogonadism is a multifaceted disorder characterized by steroidogenesis impairment through LCs desensitization to LH and deterioration of mitochondrial steroidogenic machinery (139). Therefore, we hypothesized that the expression of ORG in spermatozoa could be correlated to paternal age and its hormonal alterations. To test our

hypothesis, we evaluated *MC4R*, *GNPDA2* and *FTO* mRNA abundance of 106 sperm samples by qPCR and compare it with the age of the individuals (average age of 39.5 years old). We could not detect any correlation between the abundance of the *MC4R, GNPDA2* and *FTO* transcripts in human spermatozoa and paternal age (**Figure 12, panel A, B and C,** respectively).

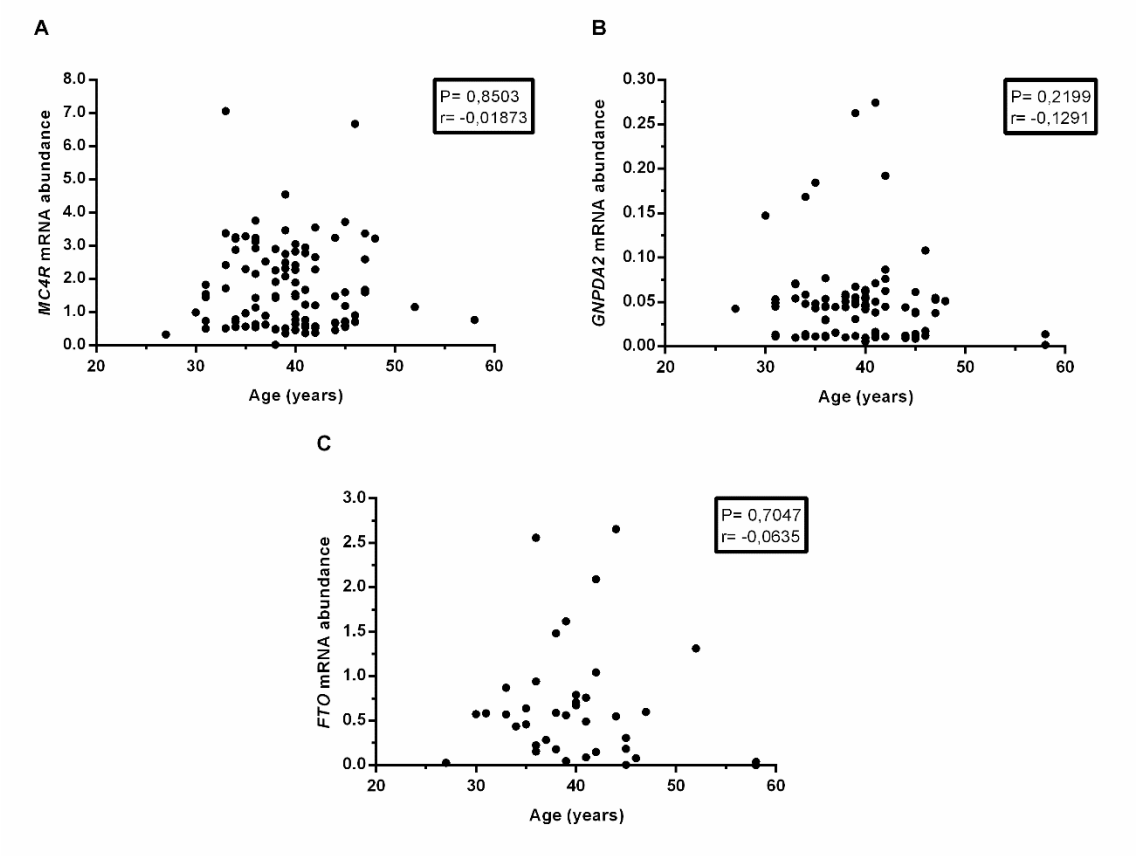

Figure 12: Pearson correlation between the expression of selected ORG in human spermatozoa and paternal age. The association between paternal age (average age of 39,5 years old) and (A) *MC4R* (n=95), (B) *GNPDA2* (n=87), and (C) *FTO* (n=35) mRNA abundance was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). No correlations between the studied parameters were found to be statistically significant.

4.5. Correlation between sperm quality and BMI, age and ORG expression

Sperm parameters are used as reliable biomarkers for male fertility potential. Previous studies reported a strong negative association between overweight/obesity and sperm quality (**Table 1**). Although our data suggest that there is no correlation between BMI and sperm parameters (specifically, total sperm count, total and progressive motility, normal morphology and viability) in the population of patients, our data showed that total (r= -0,272) and progressive motility

(r= -0,215), as well as normal morphology (r= -0,268), are negatively correlated with advanced paternal age (**Table 4**).

Table 4: Pearson correlation coefficients between sperm classical parameters and paternal BMI and age.

	Total sperm count		% Total Motility		% Progressive Motility		% Normal Morphology		% Viability	
	P	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*
BMI	0,619	-0,0501	0,254	0,116	0,252	0,115	0,104	-0,165	0,880	0,0156
Age	0,847	-0,0189	0,00530	-0,272	0,0272	-0,215	0,00660	-0,268	0,0542	-0,195

Therefore, we hypothesized that the expression of ORG in human spermatozoa could be correlated with sperm parameters. To test our hypothesis, we investigated how ORG mRNA abundance was correlated to the following sperm parameters: total sperm count, motility, morphology, viability, and hypo-osmotic swelling.

4.5.1. Correlation between *MC4R* mRNA abundance and sperm parameters

Pearson correlation between *MC4R* transcript abundance and sperm parameters revealed that the percentage of viable spermatozoa on the semen is negatively associated (r= -0.3111) with *MC4R* expression in spermatozoa (**Figure 13, panel E).** Other sperm parameters, total sperm count, total and progressive motility, normal morphology, and hypo-osmotic swelling were not correlated with the transcript abundance of this ORG (**Figure 13, panels A, B, C, D, and F,** respectively). Overall, these results suggest that *MC4R* expression may be associated with sperm quality, particularly with sperm viability.

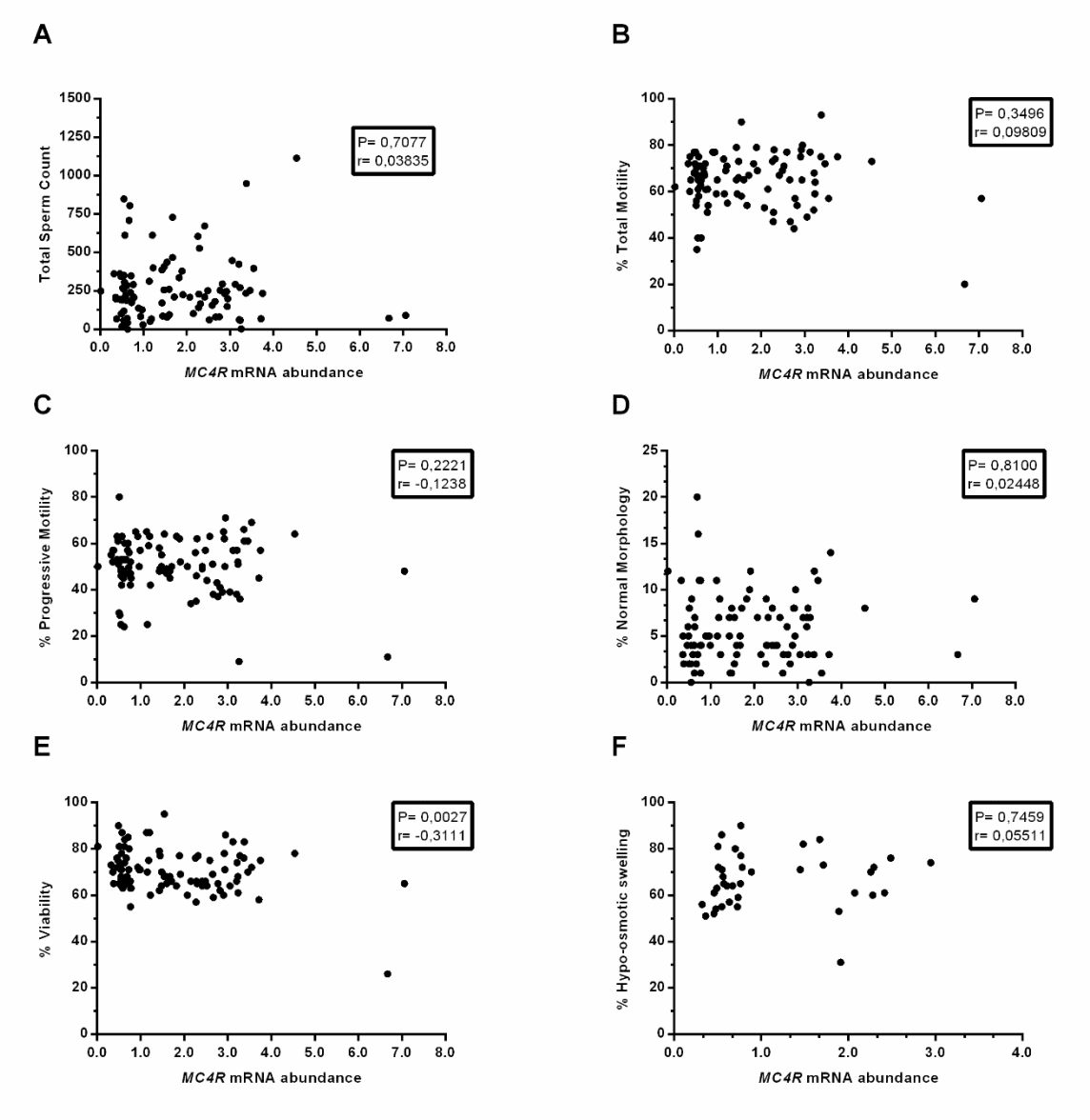

Figure 13: Pearson correlation between *MC4R* mRNA abundance and sperm parameters. The association of *MC4R* mRNA abundance and (A) total sperm count (n=98), (B) % total motility (n=93), (C) % progressive motility (n=93), (D) % normal morphology (n=98), (E) % viability (n=91), and (F) % Hypo-osmotic swelling (n=37) was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant.

4.5.2. Correlation between *GNPDA2* mRNA abundance and sperm parameters

Our results reveal that none of the sperm parameters studied, which includes total sperm count, total and progressive motility, progressive motility, normal morphology, viability and hypo-osmotic swelling, were correlated (through a Pearson correlation) to the *GNPDA2* transcript abundance, in

spermatozoa (**Figure 14**). These results suggest that the expression of *GNPDA2* in spermatozoa does not appear to be correlated with sperm quality.

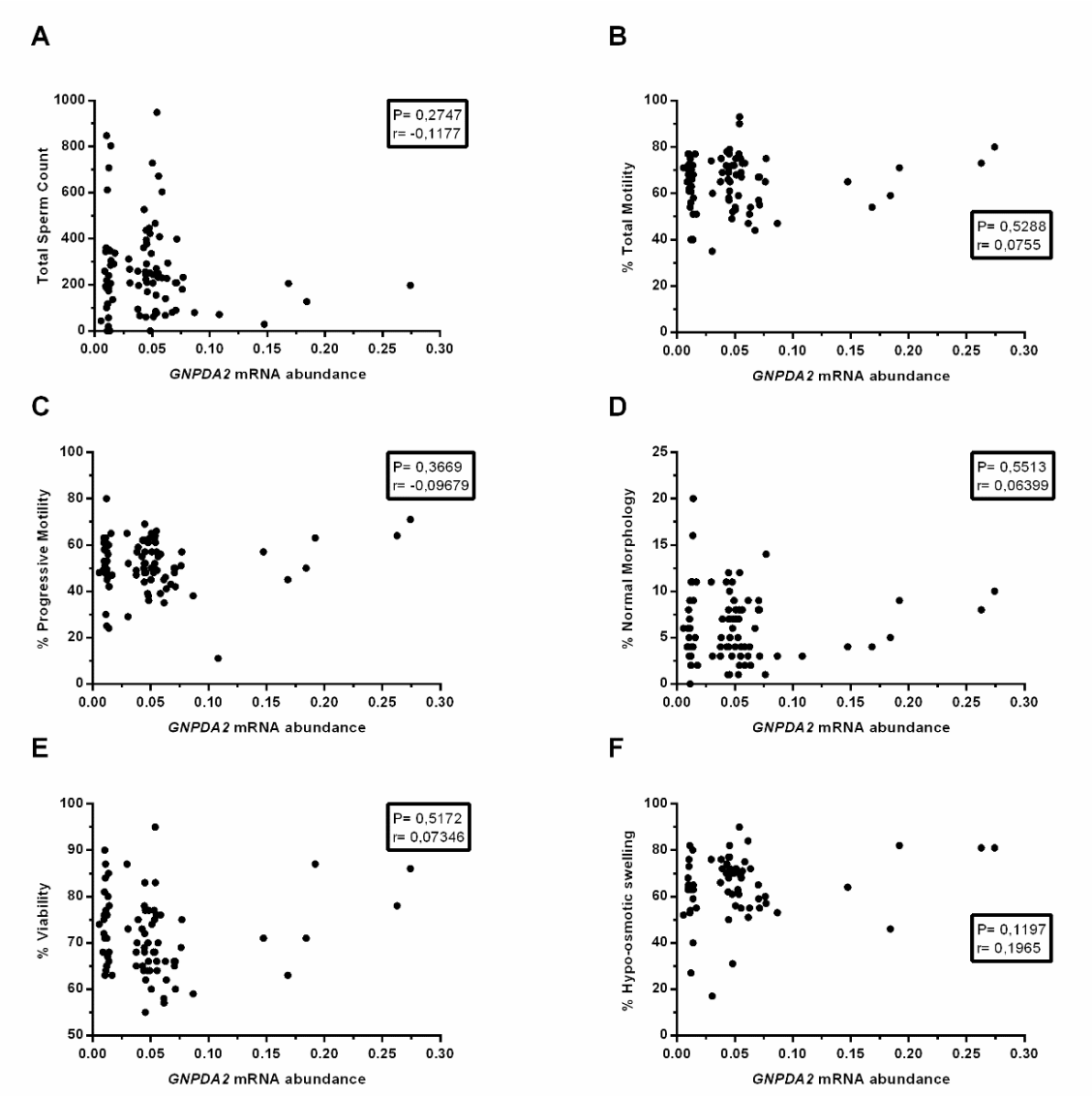

Figure 14: Pearson correlation between *GNPDA2* mRNA abundance and sperm parameters. The association of *GNPDA2* mRNA abundance and (A) total sperm count (n=88), (B) % total motility (n=82), (C) % progressive motility (n=88), (D) % normal morphology (n=88), (E) % viability (n=80), and (F) % Hypo-osmotic swelling (n=64) was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). No correlations between the studied parameters were found to be statistically significant.

4.5.3. Correlation between *FTO* mRNA abundance and sperm parameters

Pearson correlation between *FTO* transcript abundance and sperm parameters reveal that the percentage of total sperm count on the semen is positively associated (r= 0.5042) with *FTO* expression (**Figure 15, panel A**).

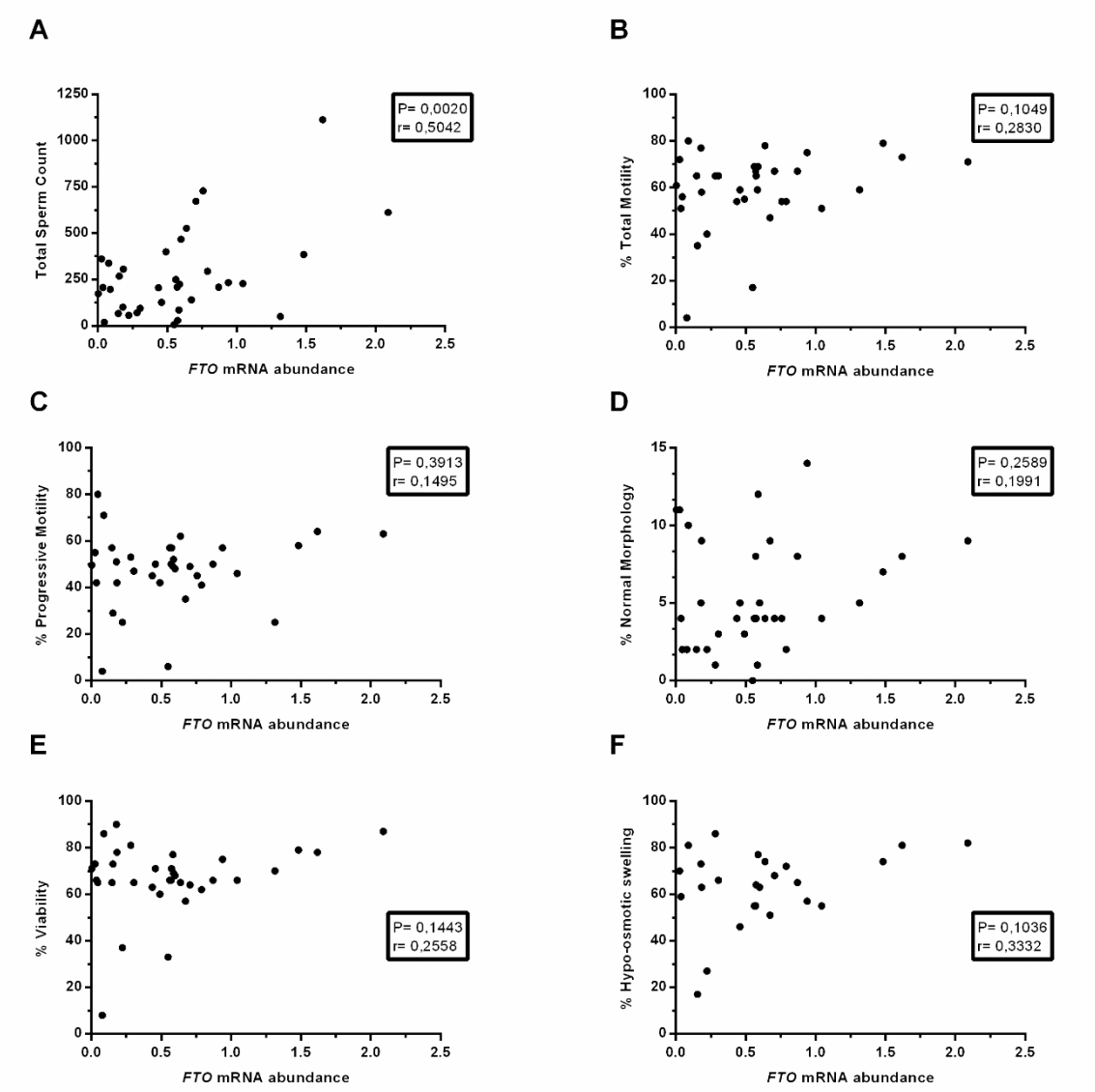

Figure 15: Pearson correlation between *FTO* mRNA abundance and sperm parameters. The association of *FTO* mRNA abundance and (A) total sperm count (n=35), (B) % total motility (n=34), (C) % progressive motility (n=34), (D) % normal morphology (n=34), (E) % viability (n=34), and (F) % Hypo-osmotic swelling (n=25) was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant.

Other sperm parameters, motility (**Figure 15, panels B and C**), morphology (**Figure 15, panel D**), viability (**Figure 15, panel E**), and hypo-osmotic swelling (**Figure 15, panel F**) were not correlated with the transcript abundance of this ORG. These results suggest that *FTO* expression is related to semen quality, specifically total sperm count, meaning that there is a possibility that this ORG is related to spermatogenesis.

4.5.4. ORG expression and spermogram classification

Accordingly to the *WHO laboratory manual for the examination and processing of human semen* (241), semen analysis is used as a clinical and research tool for investigating the male fertility status. Following strict reference values, male fertility status is classified into several categories, accordingly to the semen analysis results. Normozoospermia (NZ) diagnoses sperm parameters classified as normal. Obstructive azoospermia (A) diagnoses the absence of sperm in the semen due to male tubules obstruction or trauma. Asthenozoospermia (AT) diagnoses a low percentage of motile sperm within the semen. Oligospermia (O) diagnoses a low concentration of sperm within the semen. Teratozoospermia (T) diagnoses a low percentage of normal morphologic sperm within the semen. Oligoteratozoospermia (OT) diagnoses a low percentage of normal morphologic and motile sperm within the semen. Oligoasthenoteratozoospermia (OAT) diagnoses a low percentage of normal morphologic and motile sperm within the semen, along with low sperm concentration (241). To investigate if ORG expression in spermatozoa was associated with spermogram classification, sperm samples were grouped into 7 categories, accordingly to WHO criteria (**Figure 16**). We could not detect any association between ORG transcript abundance and any of those classifications related to sperm quality.

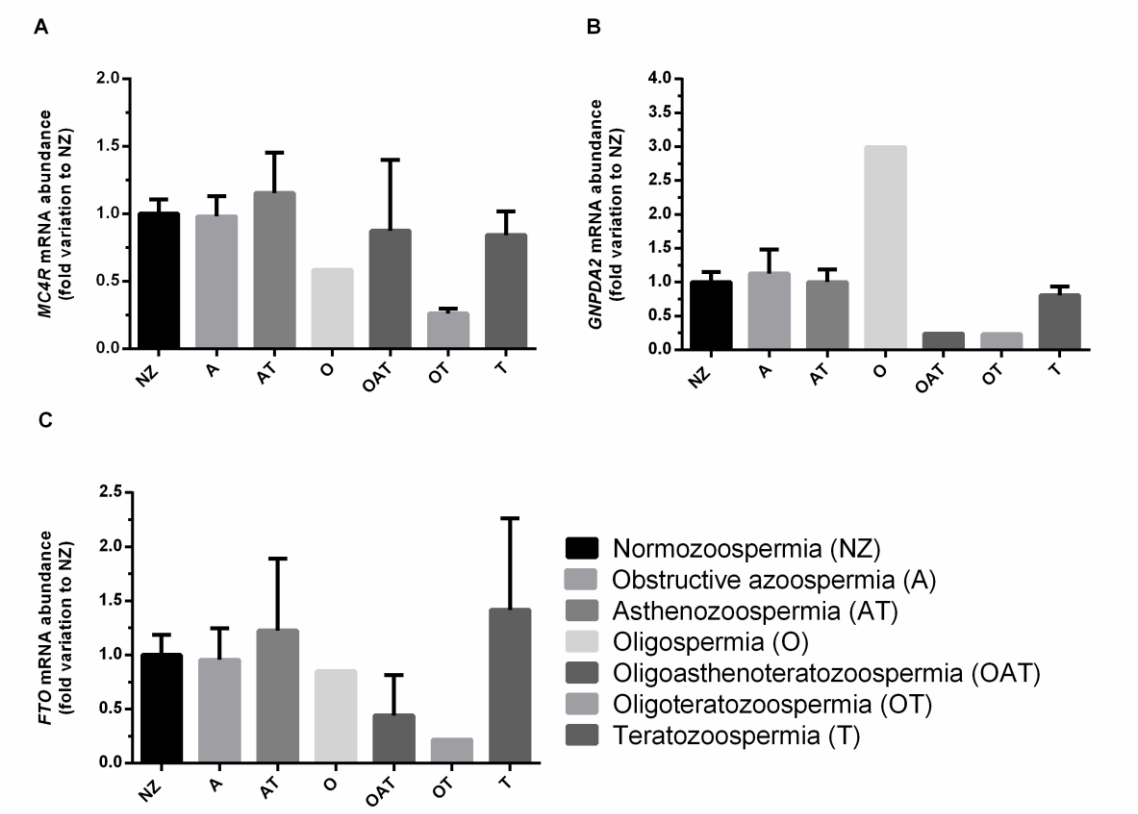

Figure 16: Effect of ORG mRNA abundance in human spermatozoa on spermogram classification. Sperm samples were grouped according to their spermogram classification. ORG expression by human spermatozoa was accessed by quantitative polymerase chain reaction. (A) refers to *MC4R* expression, number of samples in which group: NZ (n=52), A (n=14), AT (n=11), O (n=1), OAT (n=3), OT (n=2), and T (n=11). (B) refers to *GNPDA2* expression, number of samples in which group: NZ (n=45), A (n=13), AT (n=12), O (n=1), OAT (n=1), OT (n=1), and T (n=11). (C) refers to *FTO* expression, number of samples in which group: NZ (n=52), A (n=11), AT (n=14), O (n=1), OAT (n=4), OT (n=2), and T (n=14). Results are expressed as mean ± SEM. No results between the studied parameters were found to be statistically significant.

4.6. Correlation between ORG expression and embryo development

During medically assisted reproduction, the fertilization methods are chosen considering several different factors. After semen analysis, severe O, AT, T, and others are usually indicated for ICSI. This fertilization method consists of the intracytoplasmic injection of a spermatozoon into the oocyte. The reproductive history of the couple (previous pregnancies, and procedures) is also an important factor to keep in consideration (242). NZ individuals are usually indicated for IVF, which is an *in vitro* process where the oocyte is combined with the spermatozoa outside the human body,

(242). In the period between fertilization and embryo transference, embryo quality is checked at several stages. The fertilization rate is accessed 18h after fertilization. At this stage of development, embryos should present 2 pronuclei. Pos-fertilization period of 16 to 24 hours marks the beginning of the zygote cleavage stage and a two-cell embryo should presence. The zygote cleavage stage is checked every 24h, where 4-cell embryos at 48 hours and 8-cell embryos at 72 hours are considered ideal (243). In this stage, the embryo cleavage rate and high-quality embryo rate are evaluated. All embryos that fail to achieve ideal development are discarded. On day 5, the best high-quality embryos are selected rather for embryo transference or for preservation. At this stage, embryos should be at the blastocyst stage, characterized by a hollow cellular mass compose by two cellular layers: the outer layer cells, and the inner layer cell cluster (244). Since we demonstrated that some ORG, specifically *MC4R,* and *FTO,* appear to be correlated to specific sperm quality parameters, we hypothesized that the abundance of ORG transcripts on spermatozoa could be correlated to embryo quality parameters, specifically: fertilization rate, embryo cleavage rate, high-quality embryo rate and blastocyst rate.

4.6.1. Correlation between *MC4R* mRNA abundance and embryo quality

Our previous results demonstrated that *MC4R* expression, in spermatozoa, was correlated to the percentage of viable sperm in the semen (r= -0,3111), suggesting that *MC4R* expression could be associated with sperm quality (**Figure 13).** Subsequently. we hypothesized that *MC4R* transcript abundance could also be correlated to embryo quality. **Figure 17** represents the correlation between *MC4R* expression and embryo quality parameters, specifically, fertilization rate (**Figure 17, panel A**), embryo cleavage rate (**Figure 17, panel B**), high-quality embryo rate (**Figure 17, panel C**) and blastocyst rate (**Figure 17, panel D**). However, we could not detect any correlation between *MC4R* expression in spermatozoa and embryo quality rates accessed during medical assisted-reproduction treatments. Overall, these results suggest that *MC4R* expression somehow can affect sperm viability, without interfering with early embryo development at least in conditions of assisted reproduction.

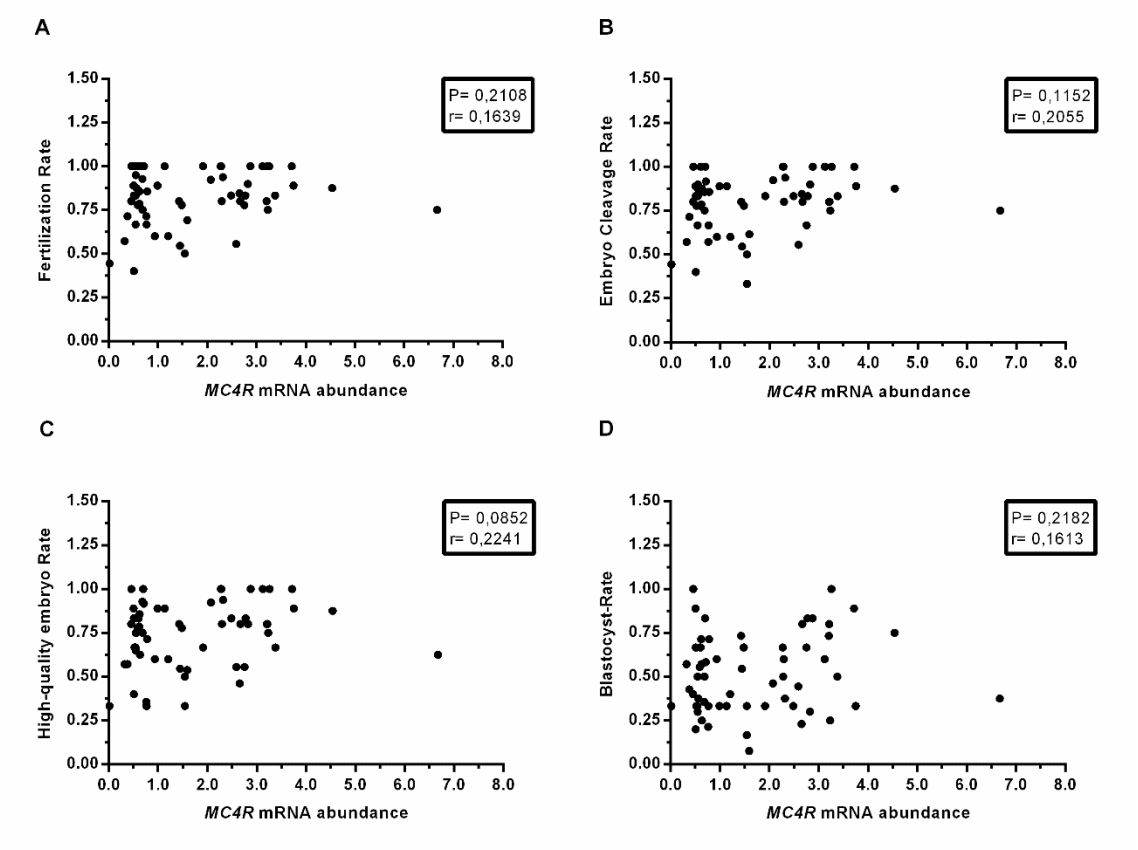

Figure 17: Pearson correlation between *MC4R* mRNA abundance in spermatozoa and embryo quality. The association *MC4R* mRNA abundance in human spermatozoa and (A) fertilization rate (n=60), (B) Embryo cleavage rate (n=60), (C) High-quality embryo rate (n=60), and (D) blastocyst-rate (n=60) was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). No correlations between the studied parameters were found to be statistically significant.

4.6.2. Correlation between *GNPDA2* mRNA abundance and embryo quality

Our previous results suggested that *GNPDA2* expression by spermatozoa was not correlated to sperm quality (**Figure 14**). Nonetheless, we investigated if the expression of this ORG could be related to embryo quality ratios by Pearson correlation (**Figure 18**). Similarly to the previous results, *GNPDA2* transcript abundance in spermatozoa was not correlated to any of the embryo quality parameters accessed, which include fertilization rate (**Figure 18, panel A**), embryo cleavage rate (**Figure 18, panel B**), high-quality embryo rate (**Figure 18, panel C**) and blastocyst rate (**Figure 18, panel D**). These results suggest that *GNPDA2* appears not to be associated with either sperm quality nor embryo early development.

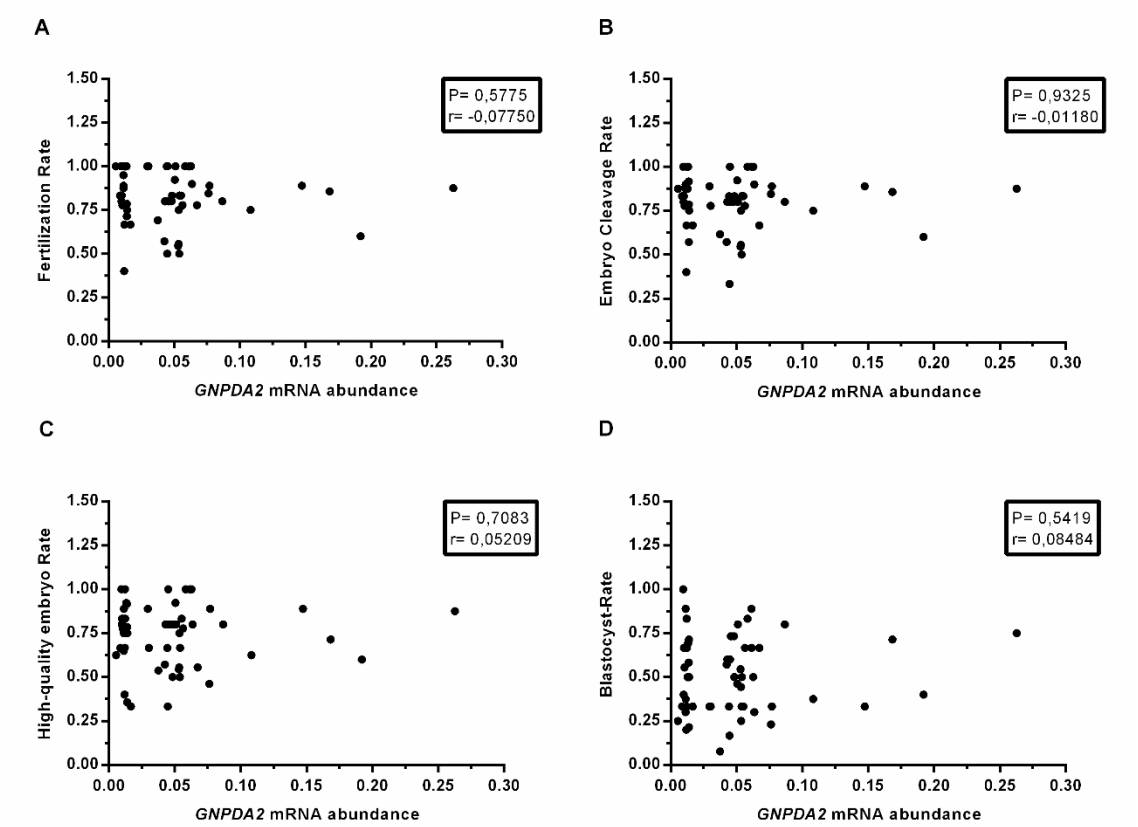

Figure 18: Pearson correlation between *GNPDA2* mRNA abundance in spermatozoa and embryo quality. The association *GNPDA2* mRNA abundance in human spermatozoa and (A) fertilization rate (n=54), (B) embryo cleavage rate (n=54), (C) High-quality embryo rate (n=54), and (D) Blastocyst-Rate (n=54) was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). No correlations between the studied parameters were found to be statistically significant.

4.6.3. Correlation between *FTO* mRNA abundance and embryo quality

Our data has led us to propose that *FTO* expression by spermatozoa could interfere with spermatogenesis since the expression of this gene was positively correlated with total sperm count (**Figure 15**). To further investigate the role of this ORG, we correlated *FTO* transcript abundance with embryo quality parameters. We could not detect a correlation between *FTO* expression and blastocyst-rate (**Figure 19, panel D**). However, we notice that fertilization rate (**Figure 19, panel A,** r= 0,4751), embryo cleavage rate (**Figure 19, panel B,** r=0,6530**)** and high-quality embryo rate (**Figure 19, panel C,** r=0,6544) were all positively correlated to *FTO* mRNA abundance within spermatozoa, suggesting that *FTO* expression in spermatozoa can act as a modulator of spermatogenesis and early embryo development.

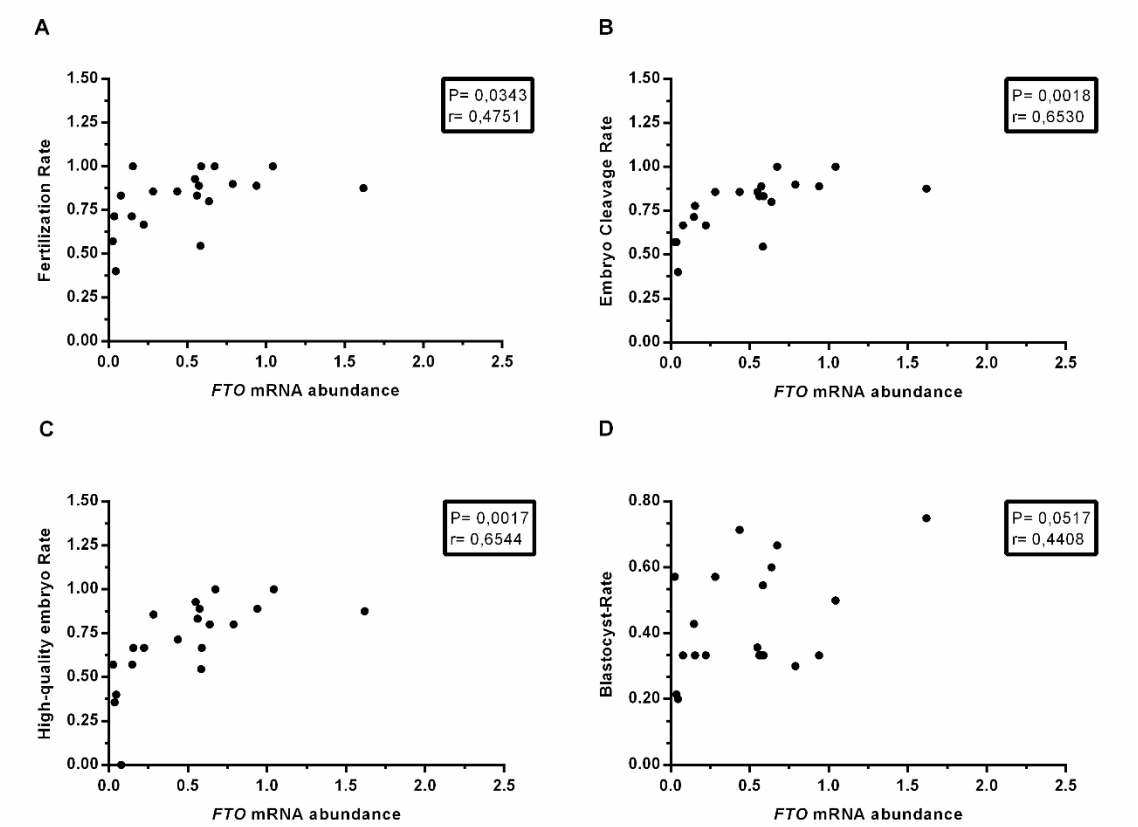

Figure 19: Pearson correlation between *FTO* mRNA abundance in spermatozoa and embryo quality. Association between *FTO* mRNA abundance in human spermatozoa and (A) fertilization rate (n=20), (B) embryo cleavage rate (n=20), (C) high-quality embryo rate (n=20), and (D) blastocyst-rate (n=20) was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant.

4.7. Correlation between ORG expression and pregnancy

From the 106 infertile couples undergoing assisted reproduction at the beginning of this study, embryo transference (of one or two embryos) was performed on 81 women. After a period of 3-7 days pos-embryo transference, serum beta-human chorionic gonadotropin (βHCG) concentration was measured. This hormone is produced by the trophoblast cells of the embryo and it is essential for promoting progesterone production and uterine vasculature angiogenesis, processes that will allow embryo implantation (245). At this point, βHCG concentration should surpass the value of 20 mIU/mL, meaning that the embryo has started the processed of implantation (246). After this point, the pregnancy is classified as biochemical pregnancy. 45 women did not become pregnant,

after embryo transference. The remaining 36 cases were classified as biochemical pregnancies. Notwithstanding, the rise of βHCG can be transient and pregnancy can fail to progress. We hypothesized that, along with the effects on sperm and embryo quality, the expression of ORG in spermatozoa could also be related to the biochemical pregnancy ratio (**Figure 20**). Although we could not find any correlation between *GNPDA2* and *FTO* expression in spermatozoa with biochemical pregnancy (**Figure 20, panels B and C**), the expression of *MC4R* was positively correlated (r=0,4502) with the biochemical pregnancy (**Figure 20, panel A**). Our data shows that *MC4R* expression could be a modulator of sperm viability, along with embryo implantation.

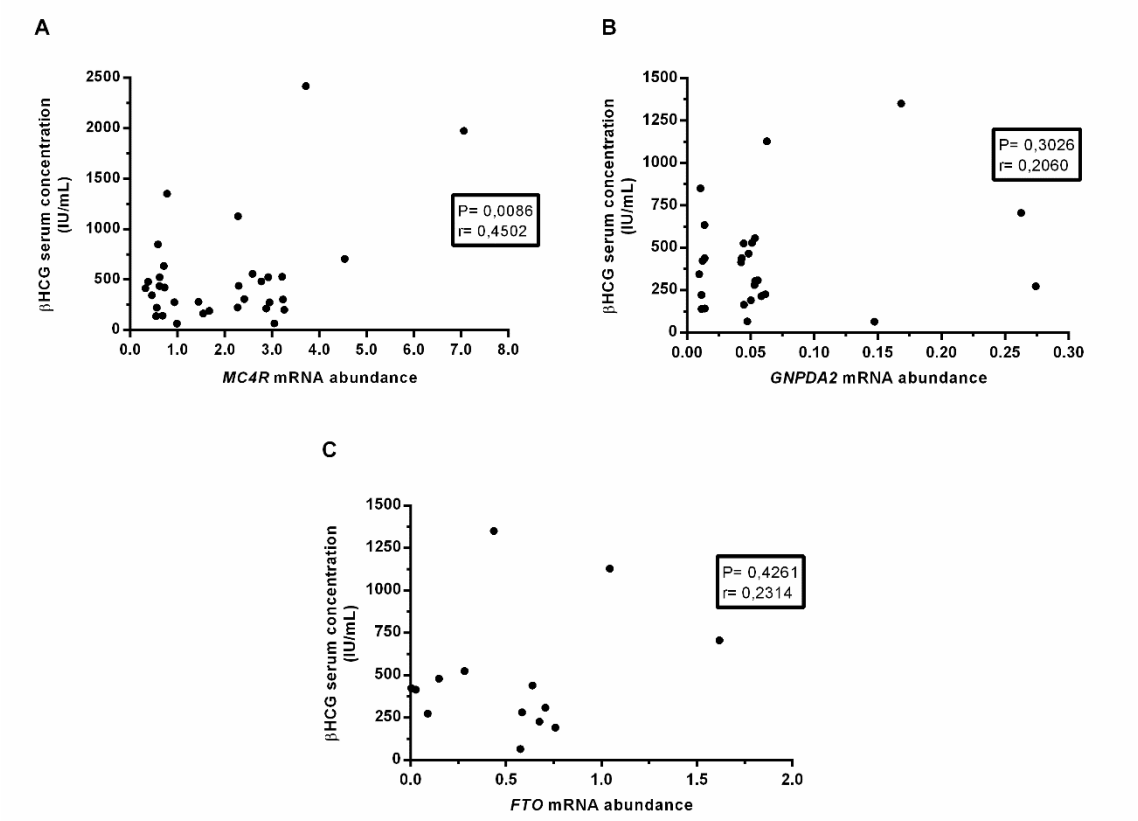

Figure 20: Pearson correlation between ORG expression in human spermatozoa and biochemical pregnancy. The association between biochemical pregnancy and (A) *MC4R* (n=33), (B) *GNPDA2* (n=27), and (C) *FTO* (n=14) mRNA abundance, in spermatozoa, was evaluated by computing Pearson correlation coefficients (r) assuming Gaussian distribution (confidence interval of 95%). All P values < 0.05 were considered statistically significant.

Until April 2019, 6 women previously classified has biochemical pregnant suffer an abortion. The remaining pregnancies evolved into clinical pregnancies defined as when the fetal heartbeat is

detected by echography. In sum, from the 81 embryo transfers performed, only 30 cases evolved into clinical pregnancies. In concordance with the previous results, we tested if the ORG transcript abundance in spermatozoa could be related to the success of medical assisted reproduction treatments, more specifically, with ongoing pregnancies (**Figure 21**).

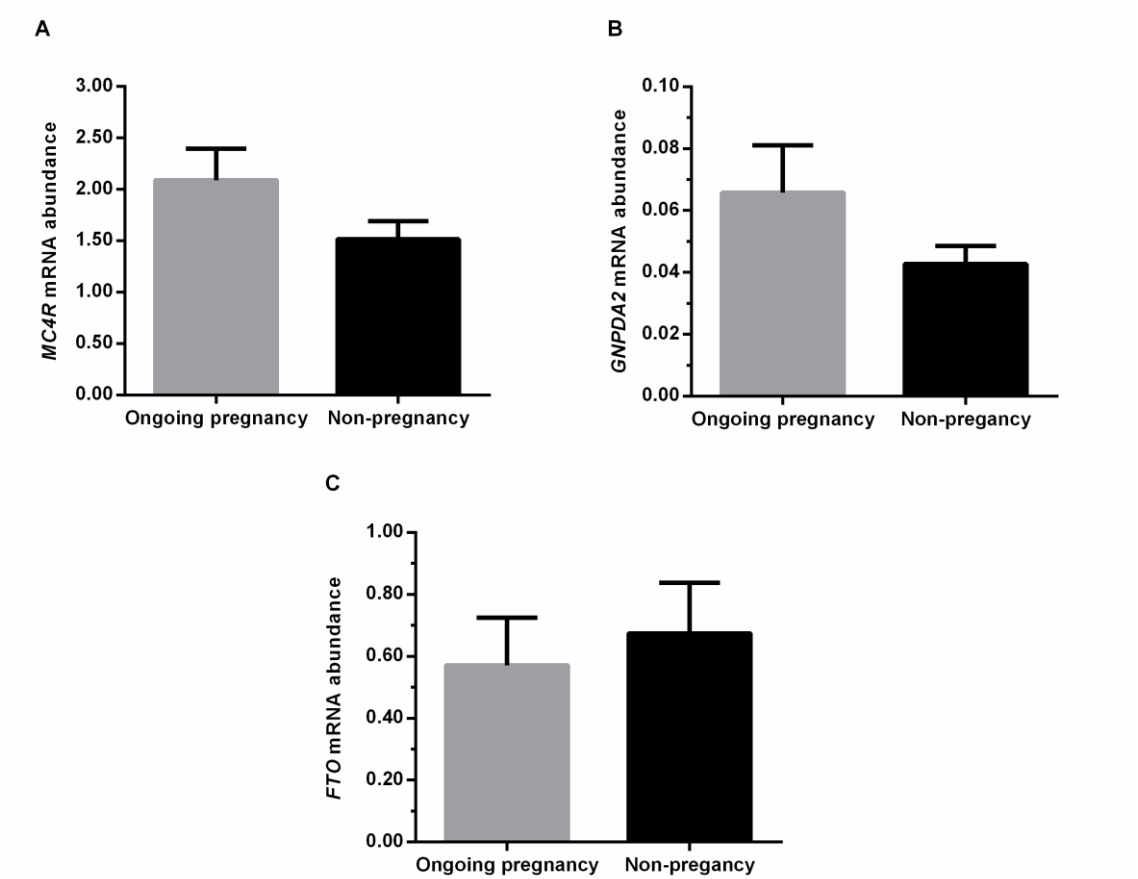

Figure 21: Effects of ORG expression in human spermatozoa and ongoing pregnancy. The expression of ORG by human spermatozoa was accessed by quantitative polymerase chain reaction: (A) refers to *MC4R* expression (Ongoing pregnancy= 27, Non-pregnancy= 46), (B) refers to *GNPDA2* expression (Ongoing pregnancy= 22, Non-pregnancy= 42), (C) refers to *FTO* expression (Ongoing pregnancy= 10, Non-pregnancy= 20). Results are expressed as mean ± SEM. No results between the studied parameters were found to be statistically significant.

However, we could not detect any correlation between the expression of ORG in human spermatozoa and ongoing pregnancies (**Figure 21**). These results suggest that the expression of these genes does not interfere with the development of pregnancy. However, these genes appear to be associated with sperm and embryo quality, suggesting that they might be important in early-

pregnancy development and, therefore, interfere with the success of medical assisted-reproduction treatments.

5. DISCUSSION

Accordingly to the WHO, noncommunicable diseases account for 71% of early-deaths at a worldwide level (247). Ischaemic heart diseases and heart stroke remain the two main causes of early-death, with both of them accounting for more than 6 million deaths, on the global health estimates from 2016. When looking into the estimates for upper-middle-income countries in the same study, we can see that liver, stomach and breast cancers, rise to the top 10 reasons for early-death. Herein, diabetes is also present, having killed 1.6 million people, only in the year of 2016 (247). It is interesting that all these diseases have in common the fact that their development is intimately associated with overweight and obesity. It is difficult to know the real impact in health of these conditions, but it is known that, in 2016, 1.9 billion people suffer from overweight and obesity (7). In addition, it is known that this number is largely underestimated and all reports highlight that they are expected to keep rising each year. Thus, it is crucial to understand the molecular pathways associated with the detrimental effects of obesity on health complications.

The negative impact of obesity on the female reproductive system has long been reported. It is known that overweight/obese women have a higher chance of developing polycystic ovary syndrome, to have more pregnancy complications, and to be more prone to suffer miscarriages in the first trimester of pregnancy (11). For those reasons, women are usually advised to lose weight when undergoing fertility treatments (248). However, the same is not true for men, even though several authors have already reported that obesity is associated to decreased semen quality, sperm DNA fragmentation, aberrant sperm mitochondrial function and other defects (80, 104-107) that may hamper the success of ART for the couples or can be associated with the transmission of genetic and epigenetic abnormalities to the offspring. Furthermore, the development of obesity is strongly associated with genetic and epigenetic causes. Studies reveal that paternal obesity can induce alterations to the DNA methylation pattern on sperm. Additionally, genes involved in metabolic regulation were reported to be epigenetic hotspots in gametes, including several ORG (173). Likewise, mutations on ORG are known to promote the development of monogenic obesity (176). Altogether, these studies suggest that paternal overweight/obesity can be transmitted to the descendants, which, as a consequence, has the potential to develop fertility complications themselves.

The aim of this project was to unveil some molecular mechanisms by which sperm may pass information towards an overweight/obese phenotype. More specifically, we hypothesized that ORG

expression, specifically *MC4R, GNPDA2, FTO,* and *TMEM18*, was associated with decreased male fertility potential. Additionally, we hypothesized that the expression levels of ORG on spermatozoa could be important to embryo development, where they played an important role in the modulation of paternal obesity effects on pregnancy outcomes.

We detected the presence of all ORG transcripts in human SCs. To our knowledge, it is the first time that *MC4R, GNPDA2,* and *TMEM18* are described in human SCs. Furthermore, we confirmed the presence of *FTO* mRNA in SCs, as it has previously been reported by Landfors and colleagues (213). We also detected the presence of protein associated with the ORG in human SCs. The presence of FTO had already been reported in human SCs through IF by Landfors and colleagues (213). To our knowledge, we are the first to report the presence of MC4R, GNPDA2, and TMEM18 in SCs. Then, we investigated the effects of treatment with obesity-related hormones on the expression of ORG in human SCs. We could not detect any alteration on the expression of ORG when SCs were treated with GLP-1. However, the same was not true for leptin and ghrelin treatments. We detected that when SCs were treated with 50 ng/mL of leptin, a concentration usually found in morbid-obese individuals, the expression of *GNPDA2* was increased. This gene encodes a deaminase enzyme that catalyzes the deamination of D-glucosamine-6-phosphate, an aminosugar, into D-fructose-6-phosphate, which can then be directed into glycolysis. Martins AD and collaborators reported that leptin was able to induce alteration on SCs metabolism (28). They reported that leptin concentrations found in normal individuals were able to promote the upregulation of glucose receptors protein expression, especially GLUT2, suggesting a higher consumer of glucose. Contrastingly, concentrations of leptin found in normal and obese individuals promoted an increase in the LDH activity, while the concentration of leptin found in morbid-obese significantly decreased the activity of this enzyme. Additionally, all concentrations of leptin decreased acetate production. With these results it is clear that leptin is able to modulate the metabolism of SCs, modulating the nutritional support of spermatogenesis in consequence (28). In this perspective, we hypnotized that GNPDA2 can participate in this metabolic shift promoted by leptin treatment promoting the entrance of aminosugars into glycolysis and promoting the synthesis of pyruvate. This process may have a compensatory nature for the detected decrease of LDH activity and acetate production in obese individuals, highlighting the metabolic plasticity of SCs, already reported in other metabolic stress conditions (28).

Martins AD and her collaborators did also study the effects of ghrelin on SCs metabolism. (65). The authors reported that ghrelin concentrations for normal-weight men caused a decrease in the

glucose consumption along with the decrease of mitochondrial complexes protein expression levels and mitochondrial potential. The maintenance of LDH activity and lactate production suggests that glycolysis is still occurring. All concentrations of ghrelin tested promoted the decrease of alanine and acetate production. Furthermore, the effects of ghrelin on SCs metabolism were more pronounced when SCs were treated with ghrelin concentrations found in severely undernourished individuals, highlighting the importance of proper-eating behavior (65). Along with these results, we detected that when SCs were treated with ghrelin concentrations found on normal-weight individuals, the expression of *TMEM18* was increased. It is difficult to know the role of TMEM18 on the SCs metabolism since little is known about its function. It is hypothesized that this protein is located on the nuclear membrane of cells, where it may control the expression of certain genes by DNA interaction with its positive charged C-terminus domain. However, this concept is still debatable (228). A study performed by Landgraf K and colleagues reveals that the knockdown of *TMEM18* in adipocytes from zebrafish larvae has reflected on the downregulation of the PPARG signaling during adipogenesis *in vivo*. (231). This signaling pathway, mainly present in adipocytes, is known to regulate the fatty-acid storage and glucose metabolism. The presence of PPAR transcripts, including PPARG have been already detected in primary germ cells and SCs from rat (249). Furthermore, Thomas K and colleagues revealed that *PPARG* was a predominant transcript in rat mature SCs and that *PPARG* knockout induced the downregulation of genes associated with lipid metabolism, such as the glycolipid transfer protein, a cytosolic protein that catalyzes the transfer of glycolipids between different intracellular membranes (249). The expression of *PPARG* in the human testis has already been reported, however, to our knowledge, the presence of this receptor in human SCs is yet to be confirmed (250). These results, together with our data, led us to suggest that *TMEM18* can participate in the regulation of the fatty-acids metabolism of SCs. Along with the results of Martins AD, our results support that *TMEM18* can be a vehicle for ghrelin effects on the mitochondrial activity of SCs, promoting the modulation of the fatty-acid metabolism of these cells.

After studying how hormonal dysregulation associated with obesity could induce alterations in the expression of ORG in SCs, and since these cells are the “babysitters” of developing germ cells, we tested if the expression of ORG was also altered in spermatozoa. As a haploid gamete cell, sperm DNA is highly condensed in spiral shape and bond to positively charged protamines, ensuring a stable conformation for DNA safe delivery to the oocyte. These characteristics lead to the arrest of almost all transcription activity since only the transcription of a few genes has been detected in

spermatozoa (251). Nevertheless, mature spermatozoa are known to storage mRNAs from pos-meiotically transcribed genes. The origin and the importance of these mRNAs are still unknown. The simplest hypothesis suggests that these mRNAs are remnants of the transcription occurring during and pre-meiosis, being merely residual (251). Some studies have contradicted this concept. It was suggested that sperm RNA could be formed in a very early-stage of spermatogenesis, where both alleles are still present. Based on the process firstly described by Abraham KA and Bhargava PM, it is possible that transcripts are actually carriers of genetic information associated with alleles that will not end-up in the final spermatozoa (252). Furthermore, some studies have already reported that sperm RNA content is important for embryo development, as Chen Q proposed after reporting that injecting tsRNA into early-healthy rat embryos alters their development (171). However, the role of paternal mRNA on the embryo and offspring development is still largely underestimated, majorly due to the small quantity of paternal RNA deliver to the embryo when compared to maternal RNA. Our results provide clear evidence that this last concept may be challenged. We identify, for the first time, the presence of ORG transcripts and respective proteins, specifically *MC4R* and *GNPDA2,* in human spermatozoa, while further confirming the presence of *FTO* mRNA, as it was reported by Landfords M and colleagues (213). To our knowledge, it was the first time that FTO protein has been identified in human spermatozoa, as well. After these results, we hypothesized that ORG mRNA expression in spermatozoa could be sensitive to the individual's BMI. However, we could not find any correlation between the expression of these selected ORG in spermatozoa and BMI, even though hormonal-alterations associated with obesity could induce alterations on ORG expression in SCs. These results suggested that obesity induces stimuli that alter the expression of ORG in SCs, however, these stimuli are not fully directly reflected in sperm. We did also investigate the effect of advanced paternal age on the expression of these selected ORG in spermatozoa. A previous study performed in murine mice revealed that older mice presented a decreased abundance of genes transcripts associated with protamination when compared to younger animals. The authors proposed that the decreased mRNA content on these transcripts could promote a deficient protamination of sperm DNA (253), which was reported to decreased sperm and embryo quality (254). However, we could not detect any correlation between the expression of these specific ORG in human spermatozoa and paternal age illustrating that their expression is not directly changed by the age of the individuals. Our results contrast with the ones reported by Rai MF and colleagues (255). The authors analyzed the expression of an ORG selection (*MC4R, TMEM18, GNPDA2,* and *FTO* were not included) with age, in the meniscus of patients

undergoing arthroscopic partial meniscectomy (n=68). They reported that BMI and age were correlated with the expression of ORG, along with the development of severe osteoarthritis, suggesting that age and obesity-related variations in ORG expression could have a role on osteoarthritis development (255). Another study investigated the influence of summed risk score (a bio-statistics variable that reflects the level of risk of developing a disease) for 32 ORG on BMI and age. The genetic analysis of a total of 1176 European-American participants revealed that the summed risk score was influenced by both age and BMI (256). Contrarily to what we reported, these studies suggest that the expression of, at least, some ORG could be influenced by BMI and age.

Since we could not detect any correlation between the expression of ORG and obesity nor age, we wonder if the expression of ORG could be indirectly correlated to sperm quality. We could not detect any correlation between semen quality and BMI, contrarily to what other authors had reported (99-101). However, we did report a negative correlation between sperm total motility, progressive motility and normal morphology with age, as other authors (257, 258) had done. While the expression of *GNPDA2* in human spermatozoa was not correlated to any sperm parameters accessed in this study, *MC4R* and *FTO* showed some interesting correlations. We reported that the expression of *MC4R* in human spermatozoa was negatively correlated to sperm viability. To our knowledge, this study is the first to correlate the expression of *MC4R* in spermatozoa and sperm viability. Additionally, a genome-wide association study performed in Holstein-Friesian bulls revealed that an *MC4R* SNP was correlated to decreased sperm motility (192), reinforcing the possibility that *MC4R* transcript is associated with sperm quality. Another study performed in human *MC4R* transfected HERK cells revealed that *MC4R* SNPs significantly induce alterations in the constitutive activity of MC4R (186, 259). The disruption of the melanocortinergic pathway by *MC4R* SNPs is known to promote food intake and, ultimately, to promote obesity (259). Furthermore, the stimulation of the MC4R is known to act as a pro-survival cell signal and it is involved in the inhibition of apoptotic proteins, such as the c-Jun N-terminal kinase (JNK). It is possible that MC4R may participate in the regulation of the balance between survival and apoptosis in developing germ cells (260, 261), however, further studies are needed the unveiled the role of *MC4R* on fertility. We did also report that total sperm count was positively correlated to *FTO* expression. To our knowledge, this is the first study to correlate the expression of *FTO* in human spermatozoa and total sperm count. However, Landfors M and colleagues have also identified two missense mutations on *FTO* that were significantly associated with sperm quality. The authors used

a factor analysis to simplify the associated between genotype and sperm quality and did not correlated the association of *FTO* mutations with specific sperm quality parameters (213). Furthermore, Yang Y. and collaborators did also report that expression of alkB homolog 5 RNA demethylase, a demethylase enzyme with a similar function to FTO, was decreased in spermatozoa from asthenozoospermic men (214). Altogether, these results suggest that FTO demethylation activity could be important to ensure sperm quality and thus mutations with potentially detrimental effects on FTO activity could induce a defective methylation process and promote, in consequence, a defective cellular differentiation and/or apoptosis (262). Similarly to MC4R, FTO could be involved in the development of infertility, in *FTO* SNP carriers, through the disruption of the survival/apoptotic signaling in developing germ cells. Notwithstanding, we could not find any correlation with the expression of *MC4R, GNPDA2,* and *FTO* in spermatozoa with infertility diagnose of the patients.

Regarding the expression of ORG in spermatozoa and embryo development, we could not detect any correlation between *MC4R* and *GNPDA2* expression and embryo quality. As it was already mentioned, the expression of *GNPDA2* was not correlated with either sperm quality nor embryo early development, suggesting that, though this gene appears to be important on SCs metabolism, it may not be important for sperm nor embryo quality. These results suggest that *GNPDA2* may play an important role, nor in spermatogenesis, but in the modulation of the nutritional support of the developing germ cells. Although the expression of *MC4R* could be a modulator of sperm viability, it appears that it may not interfere with early-embryo development. Interestingly, from all the embryo quality parameters analyzed in this work, only blastocyst-rate was not correlated with the expression of *FTO* in spermatozoa. It is known that, after fertilization, active demethylation of paternal and maternal pronucleus occurs, though both paternal and maternal genome were heavily mutilated until this point. Pulaski and collaborators proposed that the process of paternal pronucleus demethylation on the embryo was associated with the exchange of protamines for histones (263). Furthermore, they proposed that this process was not essential for embryo development since mice pups born after oocyte fertilization with round spermatids (which have no protamines) were born normal. Contrastingly, another study reveals the inheritance of a maternal allele, ten-eleven-translocation-3, which is associated with paternal pronucleus demethylation, had no effect on embryo development. However, knockout mice pups for this allele had increased morphological abnormalities (264). Although the data regarding the effects of genome demethylation on embryo development remain debatable, our results suggest that the

demethylation activity of FTO could be involved in this process, being associated with higher fertilization rates, embryo cleavage rates, and high-quality embryo rates. Withal, we must highlight that the embryo-quality parameters have several limitations, being one of the most relevant the undeniable maternal influence on which they are subjected. Because of this, maternal influence should also be considered when analyzing these results.

FTO has not any correlation with embryo implantation since it was not correlated to BP, nor with the progression of pregnancy. Like previous results, *GNPDA2* expression in spermatozoa was also not correlated to BP nor to the progression of pregnancy. Altogether, our results suggest that *GNPDA2* expression could be correlated to male reproductive health through its role in SCs metabolism, modulating the nutritional support of developing germ cells. However, it is not likely for it to have a direct role during germ cell development, thus not affecting sperm quality. Similarly, it is also not likely for it to have a direct role during embryo development, at least in the first stages of pregnancy. Interestingly, from the three ORG studied in spermatozoa, only the expression of *MC4R* was correlated to BP, along with sperm viability. To our knowledge, only a study performed by Wei R and colleagues studied the role of MC4R in embryo development (265). Herein, human *MC4R* was cloned in *Schizothorax prenanti*, a fish model. After mating, fish eggs were collected at several embryonic stages. Through qPCR, the authors reported that *MC4R* expression in early-embryos increased gradually until postnatal life. Furthermore, high expression levels of *MC4R* were detected during the embryo cleavage phase. However, the authors did also proposed that *MC4R* mRNA found in embryos was likely from the mother since unfertilized eggs did also express this gene (265). Although we found a correlation between *MC4R* expression in spermatozoa and BP, we could not detect any correlation with its expression and embryo quality parameters. A study performed in human *MC4R* transfected HERK cells revealed that the activation of MC4R inhibits JNK activity (186, 259). Members of this protein kinase family are known to be important during cell death and are involved in the development of several human diseases (260). Recently, it was also reported that JNK appears to promote trophoblast migration, a process essential for correct embryo-implantation (266). With this, it is possible that *MC4R* could be involved in the process of embryo implantation, however, its effects and importance to this process remain to be elucidated.

Chapter IV

CONCLUSIONS

6. CONCLUSION

Obesity is a complex disease with several physiological components. Due to its strong genetic component, the transmission of obesity is a problem that modern society has been struggling in the last decades. Furthermore, the detrimental effects of obesity in the reproductive system raise the concern that the offspring of obese parents may not only be predisposed to develop metabolic abnormalities themselves but also suffer from fertility problems. However, the molecular pathways associated with this process remain to be determined.

In this work, we focus on 4 genes previously correlated to the development of monogenic obesity, *MC4R, GNPDA2, TMEM18,* and *FTO* and investigated their presence and expression in human SCs stimulated with obesity-related hormones, as well as in sperm quality of men of different ages and BMI, and in embryo development of couples seeking for fertility treatment. To our knowledge, we were the first to report the presence of *MC4R, GNPDA2,* and *TMEM18* transcripts and correspondent proteins in human SCs. *MC4R* and *GNPDA2* transcripts and proteins were also identified, for the first time, in human spermatozoa. Further, although we could not identify *TMEM18* mRNA in human spermatozoa, we were able to identify the TMEM18 protein on spermatozoa through WB and IF. We reported that the expression of these genes in SCs was altered by the stimuli of obesity-related hormones. These results suggest that the hormonal dysregulation associated with obesity can indeed metabolic alterations on SCs through the expression of ORG, specifically, *GNPDA2* and *TMEM18*. These results suggest that hormonal dysregulation could induce alterations on the SCs metabolism and on the nutritional support of spermatogenesis, which could be linked to obesity-induced male infertility. Then, we hypothesized that these alterations on SCs metabolic activity could be reflected in sperm quality. We report that human spermatozoa express ORG, specifically, *MC4R, GNPDA2,* and *FTO.* We also found that the expression of *MC4R* in spermatozoa was negatively correlated to sperm viability, while *FTO* expression in spermatozoa was positively correlated to total sperm count. Furthermore, *FTO* expression was also positively correlated to fertilization, embryo cleavage, and high-quality embryo rates, suggesting that this gene may have an important role in spermatogenesis and embryo-development, probably due to its demethylation activity. Lastly, *MC4R* expression in spermatozoa was also correlated to biochemical pregnancy. Together with previous results, it appears that this receptor could be an important promoter of embryo implantation through the regulation of JNK

activity. However, the role of *MC4R* during the complex process of implantation needs to be further investigated.

In the future, to better understand the role of ORG on embryo quality, the expression of these genes in embryos of different stages of development, using animal models, should be investigated. On the other hand, it could be also interesting to investigate if obesity-related hormones stimuli could induce alterations in the expression of ORG in spermatozoa.

This work highlights the importance that paternal health has during gametogenesis and early-embryo development. Nowadays, paternal health is overlooked during medical-assisted reproductive treatments, while females' health is closely evaluated. This work highlights that paternal health, especially metabolic health, should closely follow, while metabolic disorders should be address during medical-assisted reproductive treatments.

Chapter V

REFERENCES AND SUPPLEMENTARY SECTION

7. REFERENCES

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8. SUPPLEMENTARY SECTION

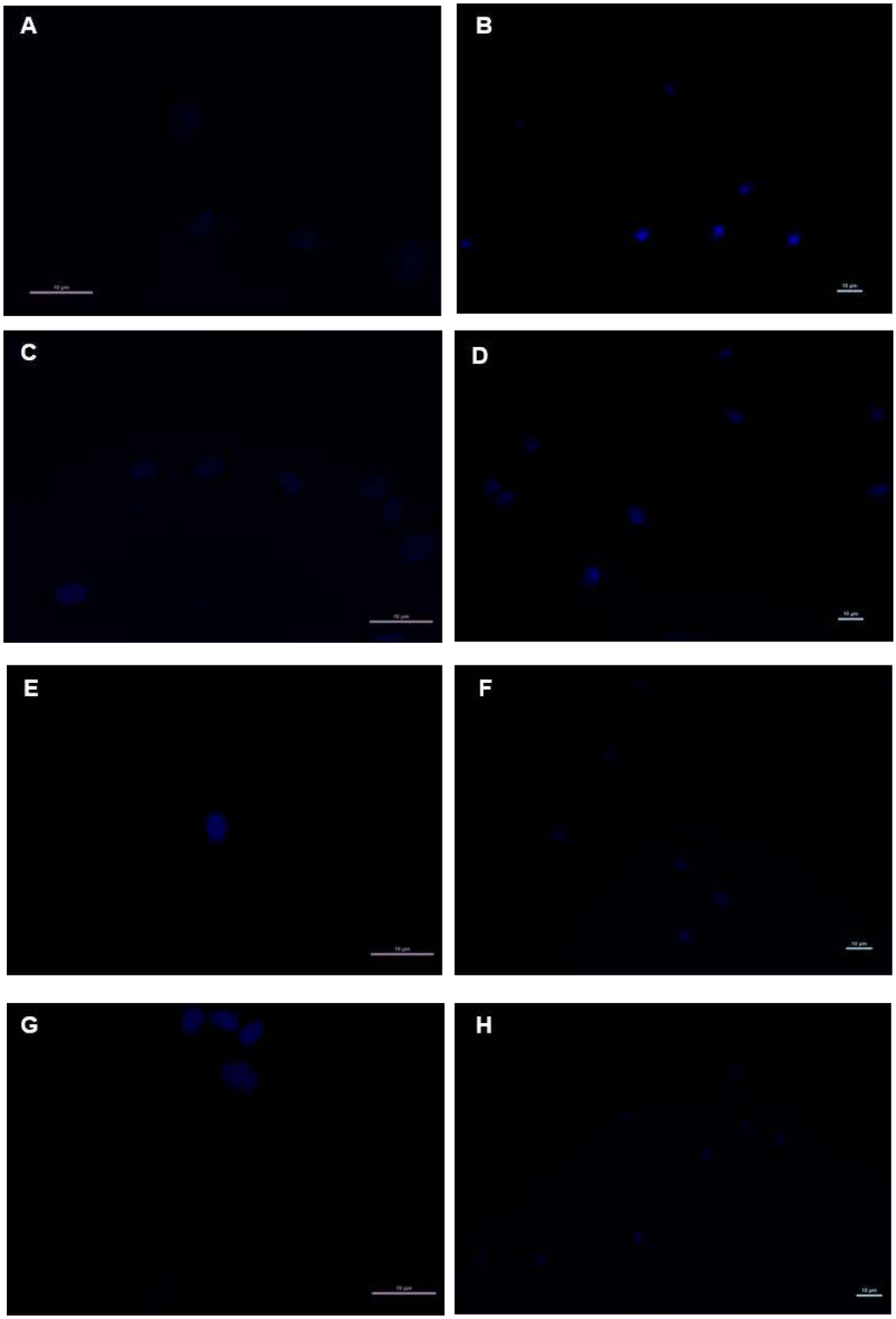

Figure 22: Negative controls of immunofluorescence staining for the studied ORG in human Sertoli Cells and Spermatozoa. Cells incubated only with Alexa Fluor secondary antibody were used as negative control. All images represent the merge results between the DAPI channel and the Alexa

Flour 546/488 channel. Negative control for MC4R in SCs (A) and spermatozoa (B). Negative control for GNPDA2 in SCs (C) and spermatozoa (D). Negative control for TMEM18 in SCs (E) and spermatozoa (F). Negative control for FTO in SCs (G) and spermatozoa (H).