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Age-related changes in human testicular function

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THE THESIS EXPLAINED

Main testicular functions are spermatogenesis and steroidogenesis. Androgen production, Testosterone (T) in particular, stimulates the differentiation of germ cells under the strict endocrine control of the pituitary gland via the two gonadotropins LH and FSH. Today we know that the reduction in serum T concentration (late onset hypogonadism) observed with age can be associated with an overall unhealthy status of the body. Moreover, the prevalence of obesity, metabolic syndrome and diabetes increases with age, and these conditions are significantly associated with hypogonadism, as assessed by sexual symptoms and low serum T levels in spite of normal LH. There is few information about mechanisms underlying androgen deficiency with age and little is known about human Leydig cell (LC) aging, in terms of number and function, mainly because of the scarce availability of the tissue.

In this study, we evaluated the age-related changes in testicular function in healthy human testicular tissues of different ages (19-85 years) obtained from heart-beating organ donors and from patients referred to the andrology clinic having normal spermatogenesis and hormone levels.

We demonstrated that aging is associated with changes in testicular morphology which are variable in different areas of the parenchyma. Changes included a significative reduction of the area occupied by seminiferous tubules coupled with an increased area occupied by interstitium, mainly composed by fibrotic tissue. In elderly donors, we noticed also peritubular thickening and the appearance of sclerotic tubules and empty tubules. Another morphological parameter evaluated was the LC micronodules frequency and size, since these aggregates composed by more than 15 LCs have been associated with a lower Testosterone/LH ratio. We did not find any correlation between age and micronodules distribution or size, pointing to a normal Hypothalamus-Pituitary-Gonad axis in our group of donors.

We found a significative reduction of both LC and Sertoli cell (SC) number with age indicating that cellular senescence generally observed in many tissues with advancing age affects also the testis.

Moreover, we evidenced a significative positive correlation between LC and SC number at all ages, which has not been described before in men.
Concerning the function of LCs, we analysed gene expression of steroidogenic pathway enzymes and other LC markers by qRT-PCR. We did not find a significative correlation between enzyme gene expression and age but a negative trend was observed for 17β-HSD3, StAR and CYP11A1 genes. In contrast, INSL3 transcript, which also reflects LC number, significantly declined in aged men, consistent with our observation that LC population size changed during aging.
Using the *in vitro* organ culture model previously developed in our laboratory, we demonstrated that three hours *in vitro* culture of both fresh and cryopreserved testis fragments allows to analyse the LC androgen production (testosterone (T), androstenedione (A), dehydroepiandrosterone (DHEA) and 17-Hydroxyprogesterone (17OHP)) secreted into the culture media and simultaneously measured by mass spectrometry both in basal conditions and under recombinant gonadotropin stimulation. We did not find any correlation between androgens secreted in basal conditions and age, indicating that LCs of the donors analysed were able to produce T, A, DHEA and 17OHP in an age- independent manner. The response to gonadotropin stimulation was found greatly variable among donors of different ages and not related to the age. The response to hCG was higher than that to LH when fresh tissue was used. Tissue cryopreservation did not alter interstitial compartment morphology but caused a significative reduction in T concentration, whereas A, DHEA and 17-OH-P levels increased, pointing to a particular vulnerability of specific enzymes to the freezing condition. These data are in line with the finding that the response to hCG was lost when the fragments were cultured after cryopreservation, reinforcing the idea that cryopreservation represents a stressor to the testicular tissue.
In conclusion, our data point to a cellular senescence of the aging human testis which is not associated to the *in vitro* ability to produce androgens. Thus, our results support the idea that LC dysfunction is largely driven by aging of the whole testicular microenvironment rather than aging of LC population alone.

INTRODUCTION

1. Testicular compartments

Testicle is the male reproductive organ in all mammals, including humans. Testicular parenchyma is divided into two functionally and morphologically different areas: seminiferous tubules and interstitial compartment, as shown in figure 1.
Seminiferous tubules occupy 60-80% of the total testis volume; they are composed by germ cells at different stages of differentiation and by a somatic cell population, Sertoli cells. Seminiferous tubules are bounded by another somatic cell type, the peritubular myoid cells. Spermatogenesis is the process by which haploid spermatozoa develop from germ cells and is the main function of seminiferous tubules. This process starts with the mitotic division of spermatogonial stem cells located close to the basement membrane of the tubules. The mitotic division of these produces two types of cells: type A cells replenish the stem cells, and type B cells which differentiate into spermatocytes. The primary spermatocyte divides meiotically (Meiosis I) into two secondary spermatocytes; each secondary spermatocyte divides into two equals haploid spermatids by Meiosis II. The spermatids are transformed into spermatozoa (sperm) by the process called Spermiogenesis. Spermatozoa are the mature male gametes.
Sertoli cells (SCs) provide structural and metabolic support at all stages of germ cell differentiation also maintaining the environment necessary for maturation via the blood-testis barrier. A single SC extends from the basement membrane to the lumen of the seminiferous tubule and supports the development of more than one germ cell. Moreover, SCs secrete a large amount of paracrine and endocrine factors affecting both seminiferous tubules and interstitial cell function and the pituitary gland control of spermatogenesis.
Interstitial compartment occupies 12-15% of the total testis volume and contains Leydig cells, immuno-system related cells such as macrophages and lymphocytes, blood and lymph vessels, nerves, fibroblasts, and loose connective tissue. The most important function of this compartment is steroidogenesis, the process leading to the production of male steroid hormones. In particular, Leydig

cells (LCs), are the cells producing androgens in the male gonad. The most important androgen is Testosterone (T). T promote the differentiation of germ cells and act as a paracrine and endocrine signalling molecule. In addition, the LCs produce a peptide hormone insulin-like factor 3 (INSL3), which is important for the testis descent and other processes.

Although anatomically separate, both compartments are closely connected with each other. For quantitatively and qualitatively normal production of sperm the integrity of both compartments is necessary.

The functions of both compartments are governed by the hypothalamus and pituitary gland (endocrine regulation). These endocrine effects are modulated at testis level by local control mechanisms (paracrine and autocrine factors).

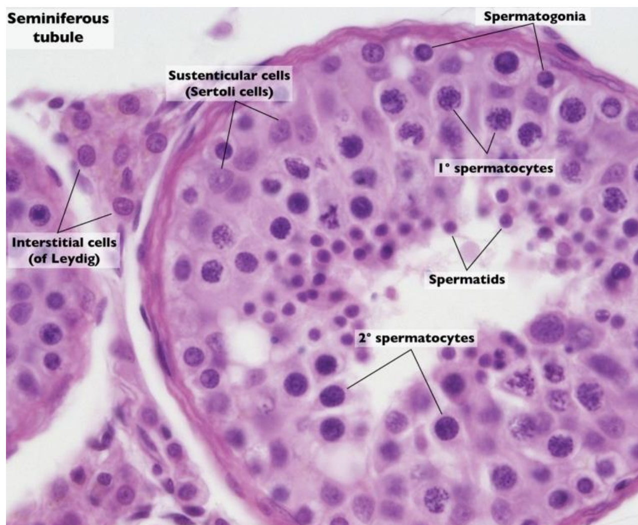

Figure 1 *Schematic representation of the testicular parenchyma architecture stained with Haematoxylin and Eosin.*

2. Leydig cells

2.1. Morphological overview

LCs were first described by Franz Leydig in 1850 (Fig 2). Morphologically, they have a large and prominent nucleus with an eosinophilic cytoplasm with numerous lipid- filled droplets rich in cholesterol (androgen precursor). The nucleus contains one to three prominent nucleoli and large amounts of dark-staining peripheral heterochromatin. Frequently some lipofuscin pigments (final endocytosis and lysosomal degradation products) and rod-shaped crystal-like structures 3 to 20 micrometers in diameter named Reinke crystals are found in the cytoplasm.

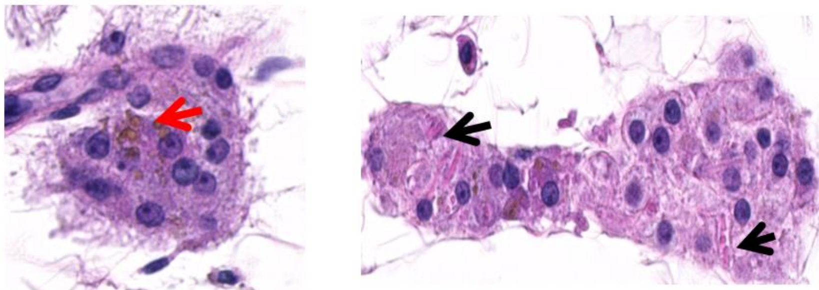

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Figure 2 *Leydig cells from an 82-year-old organ donor stained with Haematoxylin & Eosin. Lipofuscin pigments are indicated by red arrow and Reinke crystal by black arrows.*

There is a vast literature on LC development and maturation in rodents [1] [2]. These studies have greatly contributed to our knowledge of the essential steps in LC differentiation and functioning, and the factors involved in these processes. Much less information is available in humans and primates.

In contrast to rodents, in humans there are three different populations of these cells, appearing at different stages of the male development: fetal LCs (FLCs), infantile LCs (ILCs) and adult LCs (ALCs) [3].

2.2. Fetal Leydig Cells (FLCs)

The population of FLCs appears in embryonic life starting around the 7th to 14th gestation week in humans (Fig.3).
The origin of FLCs is still unclear and is the subject of some discussions and controversies. Some studies in rodents support the idea that FLCs originate from the migration of cell populations of the adjacent mesonephros and coelomic epithelium[4]. Another argument is that the FLCs share a common origin with adrenocortical cells [5]. The differentiation and the development of the FLCs is known to depend on several factors, including steroidogenic factor 1 (SF1), Desert Hedgehog (DHH), Platelet-Derived Growth Factor (PDGF), insulin-like growth factor 1 (IGF1), Hepatocyte growth factor (HGF), and Aristaless related homeobox (Arx) secreted into the microenvironment, suggesting that the differentiation of FLCs is regulated by a network of signalling pathways originating from Sertoli and other somatic cells [1].
In humans, before the start of production of LH by the pituitary gland, human chorionic gonadotropin (hCG) promotes the differentiation of FLCs during pregnancy. In the mouse, FLCs produce androstenedione because they lack 17β hydroxysteroid dehydrogenase (17βHSD) enzyme, and the final conversion to T is carried out by the fetal Sertoli cells [6]. In both rodents and humans, the FLCs also produce INSL3, a peptide hormone which is essential to induce the descent of the testis into the scrotum. Importantly, INSL3 is becoming a very useful constitutive biomarker of LC functionality [7].
Based on the emerging evidence from morphology and cell tracing studies using genetic murine models, two hypotheses concerning the fate of FLCs are supposed: (1) the FLC population undergoes involution or degeneration after birth and is replaced by ALC in adult testes; (2) the FLC population survives and becomes part of the adult LC population [1].

2.3. Infantile Leydig cells (ILCs)

Humans have an infantile LC population that appears 2-3 months after birth and declines shortly thereafter. The neonatal period in male development is characterized by an acute rise in serum T, which peaks 2 to 3 months after birth and contributes to a "minipuberty" event. In boys, mini puberty contributes to the masculinization of the brain [8].

2.4. Adult Leydig cells (ALCs)

Studies on rodents showed that the differentiation process of the ALC population can be divided into four steps: stem cells, progenitor cells, immature cells, and mature cells, based on developmental changes in cell morphology and in steroidogenic capacity.

Stem Leydig cells (SLCs) are spindle-shaped cells already seen at postnatal day 7 in the peritubular layer [9]. These cells do not express any of the LC-specific markers, including 3β-hydroxysteroid dehydrogenase (3βHSD) [10, 11]. Under the influence of various factors (DHH, PDGF), the SLCs proliferate and differentiate into progenitor Leydig cells (PLCs). The PLCs contain only small amount of smooth endoplasmatic reticulum (SER), and are identifiable by the expression of LHR and steroidogenic enzymes such as 3βHSD, cytochrome protein P450scc, and P450c17 (with 17α-hydroxylase or 17,20 lyase activity). The PLCs express low levels of the steroidogenic pathway enzymes and lack 17βHSD. Consequently, androstenedione is quickly metabolised to androsterone [11]. PLCs are highly proliferative and active in the cell cycle. Then, PLCs acquire numerous lipid droplets, and the SER compartment expands greatly because of the action of some growth factors (IGF-1, PDGF-α, T3), giving rise to immature Leydig cells (ImLCs). ImLCs express high levels of 3βHSD and LHR, as well as P450scc, and P450c17 cells [11]. The content of lipid droplets decreases when these cells mature into ALCs. This transition may reflect a change in the intracellular source of cholesterol used in steroidogenesis. Esterified cholesterol from lipid droplets may be the predominant source in

ImLCs, while in the subsequent development phases it is derived from lipoprotein cholesterol in serum or synthesized de novo [12]. In ALCs, 17βHSD begins to be highly expressed, catalysing the conversion of androstenedione into T to complete the androgen biosynthetic pathway. ALCs are large cells that are mainly localized close to blood vessels in the interstitium. They have high steroidogenic enzyme activity, and secrete T as a final product. ALCs secrete large amount of INSL3. In vitro experiments show that INSL3 induces T secretion by mouse LCs through a cyclic adenosine monophosphate (cAMP) dependent mechanism [13].

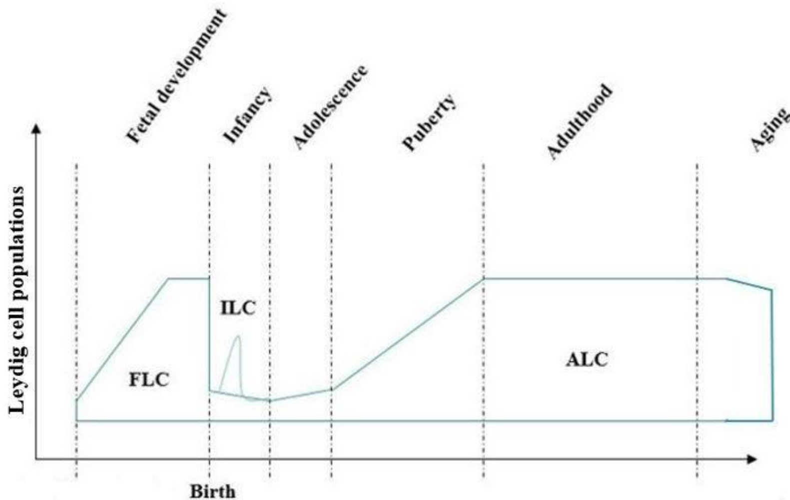


Figure 3 *Human Leydig cell populations: fetal (FLC), infantile (ILC), adult (ALC). Modified from Griswold et al. 2009 [5].*

2.5. Peaks of Testosterone production during male development

As a consequence of the three LC population, in humans there are three peaks of T concentration during male development (Fig.4).
The first peak is around the 14th -18th gestational week. It is carried out by FLCs and contributes to the masculinization of the foetal embryo, including differentiation of the Wolffian duct, development and morphogenesis of the male genital tract and external genitalia,

gonadogenesis, formation of ALC precursors, and potentially sexual dimorphism of the brain.
The second peak happens at the 2nd-3rd month after birth, is carried out by ILCs and cause the mini-puberty event associated with the masculinization of the brain.
The third and final increase in serum T concentration is carried out by ALCs at puberty determining the male secondary characters (e.g., tone of voice, musculature, aggression, growth of hair, growth of the penis, testicles, and prostate). T concentration starts a slow steady decline by the age of 40 onwards (see paragraph 5).

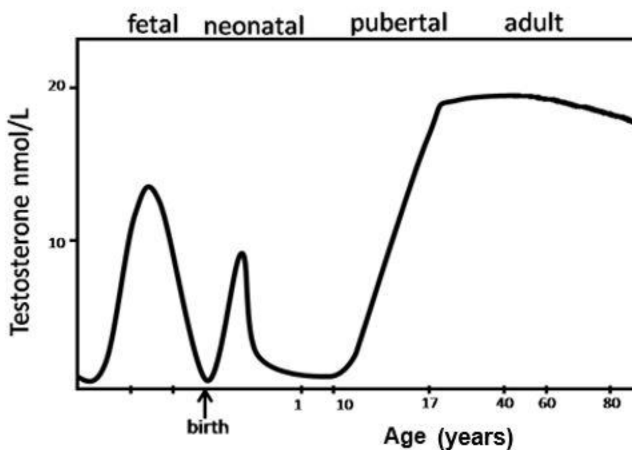


Figure 4 *Peaks of Testosterone concentration in serum during human male development.*

2.6. Leydig cell micronodules

LCs are usually found in the interstitial compartment as groups of 2-8 cells in healthy men. The proportion of testicular tissue occupied by LCs increases with decreasing spermatogenic capacity. Groups of more than 15 LC, better known as LC micronodules, are a common finding in testicular biopsies from men with impaired spermatogenesis: spermatogenic arrest, Sertoli cell only, mixed pattern of impaired spermatogenesis, complete spermatogenesis in combination with elevated FSH. [14]. Moreover, micronodules are associated with a decreased ratio of testicular hormones to

gonadotrophins (T/LH, T/FSH). Thus, the presence of micronodules is interpreted as a marker of testicular failure in men. There is no information about micronodules distribution during aging and if there is an association with the T decline observed in elderly men.
Interestingly, despite morphological differences between normal LCs and LCs within micronodules present in disorders associated with testicular dysgenesis, no significant differences in global gene expression were observed between the two groups [15].

3. Steroidogenesis

3.1. Testicular steroidogenesis

The hypothalamic-pituitary axis is involved in the regulation of testicular T production. In particular, the hypothalamus produces the hypothalamic gonadotropin-releasing hormone (GnRH) that acts on the gonadotropic cells localized in the adeno-hypophysis (the most ventral part of the pituitary gland) which produces LH and FSH. Since GnRH secretion is pulsatile, gonadotropin release also occurs in discrete peaks, more evident in the case of LH, due to its shorter half-life in circulation compared to FSH.
LH and FSH exert their function via specific receptors. In particular, LH signal is received at the plasma membrane level in LCs through LHCGR (LH receptor). LHCGR is a typical G protein-coupled receptor. When LH binds at the extracellular domain of the receptor the G protein activates the adenylate cyclase. The result is an increase of cAMP concentration which activates the protein Kinase A [16]. The cAMP/PKA pathway induces the phosphorylation events necessary for the expression of proteins involved in steroidogenesis, such as steroidogenic acute regulatory protein (StAR) and steroidogenic enzymes (P450c17, 3βHSD, and 17βHSD) as well as the release of cholesterol from lipid droplets or from plasma membrane of LCs. StAR expression triggers the cholesterol movement to mitochondria outer membrane and its translocation to the inner mitochondria membrane. Numerous studies have suggested that cholesterol translocation into mitochondria is mediated by a complex of proteins

(transduceosome), the most important of them are StAR, translocator protein (TSPO) and voltage dependent anion channel 1 (VDAC1) [17] [2].
There are two classes of enzymes involved in T biosynthesis, the cytochrome P450 proteins (CYP11A1 and CYP17A1) and the hydroxysteroid dehydrogenases (3βHSD, 17βHSD) [18] [19].
Inside mitochondria the first reaction in androgen biosynthesis takes place which is the conversion of cholesterol into pregnenolone catalysed by P450scc (cholesterol side-chain cleavage enzyme aka CYP11A1). Pregnenolone can spreads through the mitochondrial membranes and is further metabolised by enzymes associated with the smooth endoplasmic reticulum (SER) (Fig.5).

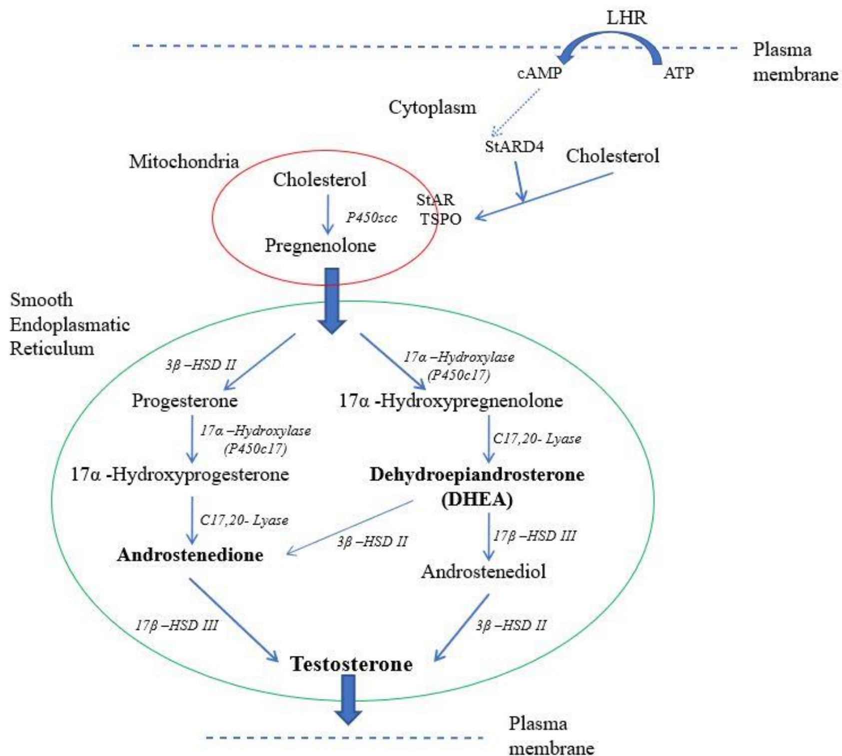


Figure 5 *Signalling pathway to Testosterone production in human LCs.*

Two steroidogenic pathways for T conversion are known in SER, the Δ^5 and Δ^4 (Fig. 6).
In the Δ^5 pathway, pregnenolone undergoes 17α-hydroxylation to 17α-hydroxypregnenolone and scission of the C17-C20 bond to yield dehydroepiandrosterone (DHEA) both catalysed by P450c17 which has two different activities: 17α-hydroxylases and 17,20 lyase respectively. DHEA is converted to androstenediol by 17β-Hydroxysteroid Dehydrogenase 3 (17βHSD3) and T biosynthesis is completed by 3β-Hydroxysteroid Dehydrogenase/Δ^5 to Δ^4 Isomerase 2 (3βHSD2).
In Δ^4 pathway, pregnenolone is converted into progesterone by 3βHSD2 that is the first biologically important steroid in the pathway. Progesterone is converted in 17α-hydroxyprogesterone and then in androstenedione (A) by P450c17 and finally in T by 17βHSD3.
Both pathways are active in the testis but even if human P450c17 17α-hydroxylates with equal efficiency pregnenolone and progesterone, the lyase activity of this enzyme is 50 times more efficient in the conversion of 17α-hydroxypregnenolone to DEHA. DHEA is preferentially converted in androstenedione by 3βHSD2 and then in T by 17βHSD3 [20].

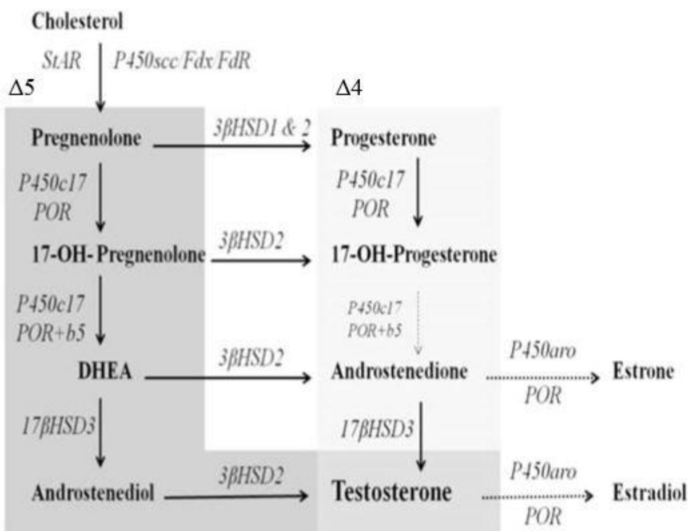


Figure 6** Leydig cells steroidogenic pathways: Δ^5 pathway is represented in dark grey and Δ^4 pathway is represented in light grey. Belli, S. et al, 2016.* ***[21]

3.2. Androgens

Androgens are produced by both testicles and adrenal glands.
T is the most important androgen and the main secretory product of the testis. T acts both as an endocrine, paracrine and autocrine factor. As a paracrine factor, it plays a pivotal role in stimulating the differentiation of germ cells, acting on Sertoli cells, myoid cells and peritubular cells through the androgen receptors (AR), that are absent in germ cells. T acts also on LCs themselves (autocrine action) as a regulator of the steroidogenic pathway. As an endocrine factor, it acts on several tissues such as prostate, muscles, bones, brain, skin where it is metabolized in its most active form 5α-Dihydrotestosterone by the action of 5α-reductase. Circulating T acts also as a negative feedback on the LH production from the hypophysis.
While serum T concentration derives mostly from testis, DHEA and A are the principal products of adrenal gland with a little contribute from the gonads. Circulating levels of DHEA are higher than other steroid hormones and can be described as a “human molecule” because other species have lower concentrations, especially the rodents [22]. The physiological role of DHEA remains unknown, but DHEA supplementation has been proven of benefit in typical deficient states such as adrenal insufficiency, autoimmune disorders, major depressive illnesses as well as to treat shock, trauma and haemorrhage and for the wellness of the skin. It has been demonstrated that DHEA and A have low capacity to bind and activate androgen receptors (AR) hence, they are androgen precursors [23]. A function is still unknown.
Also 17-hydroxyprogesterone and progesterone are secreted by the testis but their function is still object of investigations in male. Progesterone receptors have been found in some peritubular cells and on spermatozoa but a direct effect on testicular function was not found. [24-26].

4. Insuline-like factor 3 (INSL3)

Insulin-like peptide 3 (INSL3), is a member of the relaxin-insulin family of peptide hormones and it is one of the major secreted production of LCs of the mature testis. It is the specific ligand of the relaxin family peptide receptors (RXFP2, also known as LGR8), a class of G-protein coupled receptors which in the fetus are expressed in the gubernacular ligament promoting testicular descent, in cremaster muscle and in the Wolffian duct while in the adult, RXFP2 is expressed on post-mitotic germ cells, as well as in LCs and in the epididymis [27]. It has been demonstrated that INSL3 produced by LCs can cross the blood-testis barrier to get to seminiferous tubules and epididymis acting as antiapoptotic factor on germ cells [28]. Moreover, as an endocrine hormone, INSL3 probably modulates bone metabolism, since individuals with a dysfunctional mutated RXFP2 receptor exhibit significant osteopenia and osteoporosis [29].

INSL3 is constitutively expressed in a differentiation-dependent manner correlated with the postnatal development of LCs [30] and it is released in an unregulated manner as soon as it has been made. For this reason, is proposed as a less ambiguous measure of LC functionality, because differently from T, is not acutely regulated by the hormones of the HPG axis and its concentration reflects LC differentiation status and their absolute number [7, 31].

Moreover, INSL3 is not sensitive to gonadotropin stimulation in normal men, but declines markedly in response to gonadotropin deprivation. After suppression, INSL3 was responsive to hCG 4 days after administration. After long-term suppression, INSL3 did not recover to the same degree as T, suggesting that INSL3 is more sensitive to LC impairment than T [31].

Low levels of INSL3 are observed in many pathological conditions such as infertility, obesity, Klinefelter syndrome [32]. INSL3 shows also a clear and linear age-dependent decrease, probably reflecting a gradual loss of LC function and number with age [28].

5. Aging and androgen deficiency

5.1. Testosterone decline with age

It has been demonstrated in several papers that serum T levels decline with aging in men [33-36]. This decline is slow and progressive (1-2 % reduction rate/year), it starts around the 40th year of a man life and occurs even in the absence of disease (Fig. 6). The decline in serum T concentration is accompanied by increased or unchanging levels of LH [28], suggesting that the relative unresponsiveness of the LCs to LH, rather than, or in addition to, defects in the HPG axis, plays a primary role in this age-related reduction of T [37-40]. Moreover, there are very few and controversial data in humans regarding the LC number and if it changes with age. In the past years, many studies have demonstrated a decrease in the number and volume of LCs in older men using histometric analysis of perfused testis [41, 42]. However, these findings are in contrast with recent works that have demonstrated by quantitative stereological methods that the total number of LCs did not change during aging [43]. These conflicting results may be explained principally by the difference in counting approaches used as well as in the differences in subject characteristics.
To date, whether the serum T decline in healthy men is due to a reduction in LC number, a reduction in their functionality or a combination of the two parameters, is still poorly understood.

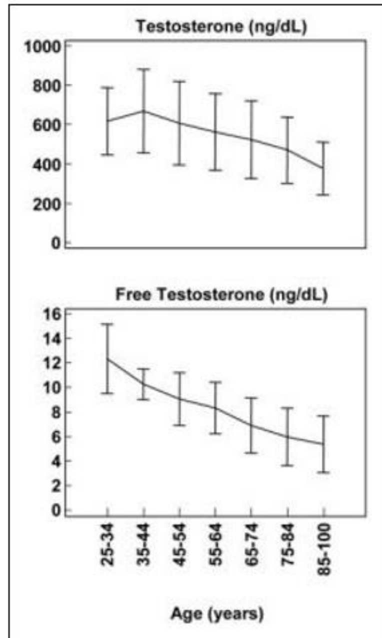


Figure 6 *Total and free testosterone reduction in serum during human aging. Kaufman et al.2005 [44].*

5.2. Male hypogonadism

Reduced serum level of T, commonly named hypogonadism, can occur in both young and aging men.
Hypogonadism can be classified according to (i) the level of HPG axis that is primary affected (primary, secondary or combined), (ii) the testicular cell population initially impaired (whole testis or dissociated testicular dysfunction); (iii) the period of life when the gonadal function begins to fail (fetal or postnatal). Primary hypogonadism (PH) is referred to a condition where LCs are initially affected. Lacking the negative feedback of T to the HPG axis, PH is characterised by an elevation of circulating LH and FSH levels mainly after puberty (hypergonadotropic hypogonadism). The only therapy that can be used is a T replacement therapy (TRT) [45]. Secondary hypogonadism (SH) is characterized by testicular failure owing to a central disorder affecting the release of GnRH hormone. The result is that patients have normal testes but a low

concentration of circulating LH and FSH (hypogonadotropic hypogonadism). Treatment options can include T, gonadotropins or GnRH treatments [45].
The clinical consequences of hypogonadisms (secondary, primary or combined) depend on the period of life in which the function of testis begin to fail. Fetal hypogonadism typically result in DSD, presenting ambiguous genitalia, cryptorchidism or micropenis. At puberty age, hypogonadism is characterized by the absence or the arrest of pubertal development, owing to androgen deficiency, secondary sexual characteristic undeveloped, testicular volume reduction, lack or arrest of spermatogenesis.
The androgen deficiency that occurs with aging is named late onset hypogonadism (LOH or andropause) and is characterized by many metabolic disorders including low libido, erectile dysfunction, infertility, gynecomastia, hot flashes, low energy, sleep disturbance, depressed or labile mood, impaired cognition, osteoporosis, and loss of muscle mass or increased body mass index (BMI) [46] (Fig. 7). Together these symptoms constitute an impairment of individual health and quality of life. LOH is more difficult to diagnose because T serum concentration can be influenced by other comorbidities which must be considered in the assessment of androgen status of an old man (obesity, alcohol, smoking, drugs, illnesses). The normal reference range for serum total T level in adult men is approximately 300-1000 ng/mL. The Endocrine Society Guidelines defined LOH as the presence of three sexual symptoms (decreased frequency of morning erections and sexual thoughts and erectile dysfunction) in combination with total T less than 11 nmol/ liter and free T less than 220 pmol/liter [47].

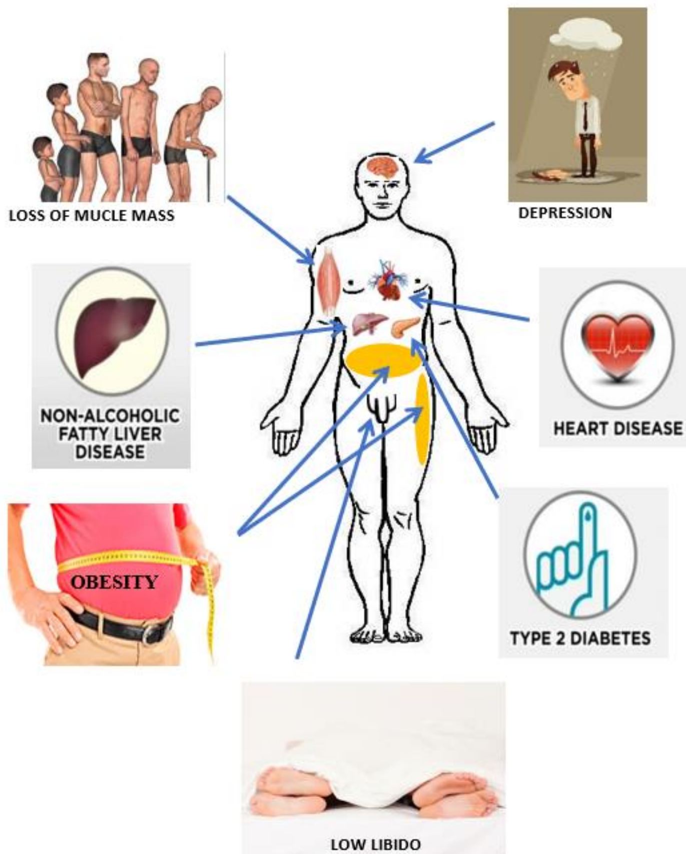


Figure 7 *The metabolic impairments due to Testosterone deficiency in elderly men.*

5.3. Side effects of long-term replacement therapies

Administering exogenous T, known as T replacement therapy (TRT), reverses many of the symptoms of low T. T preparations now a day in use are injections, scrotal and non-scrotal transdermal patches, oral, buccal and gel preparations and are widely diffused.

However, recent studies, suggested that the long-term treatments may increase the risk of cardiovascular diseases and prostate cancer in older men [48-51] (Fig. 8).

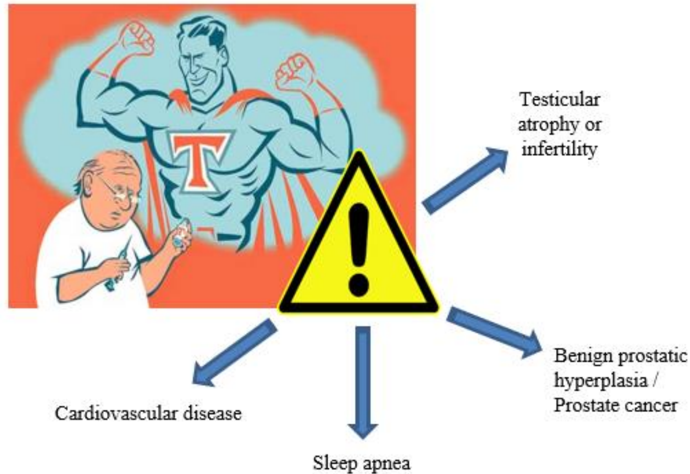


Figure 8 *Side effects of long-term treatments with Testosterone replacement therapies.*

Although there is a general agreement that TRT in young hypogonadal men is relatively safe and of benefit, exogenous T in the long term suppress LH production, resulting in reduced LC T production and a suppression in spermatogenesis. The recovery of spermatogenesis after finishing the treatment often requires 6-15 months or more [52].

There are other methods to increase serum T, such as the administration of hCG, LH or aromatase inhibitors but they are typically ineffective in men with primary hypogonadism or LOH [2].

There is no information about mechanisms underlying T decline with age, the better understanding of whom should improve a lot the quality and safety of treatments.

6. About mechanisms underlying Testosterone decline: what we know from animal models

6.1. Studies on Brown Norway Rat

As in men, aging in Brown Norway rats is characterized by reduced serum T and unchanged or increased LH levels. Those are the reasons why this animal was selected as the best animal model to study mechanisms underlying T decline together with their longevity.
The number of LCs in elderly rats was found unchanged in contrast to a significant reduction in Sertoli cell (SC) number.
Elderly rat LCs showed a reduction in LH-stimulated cAMP production, as well as a slower cholesterol uptake into mitochondria and a reduced expression and activity of steroidogenic pathway enzymes both *in vivo* and *in vitro* [36].
Other age-related changes have been suggested to indirectly impact steroidogenesis, including decreased Leydig cell cholesterol synthesis and mobilization [53], decreased autophagic activity of the cells [54], increased nitric oxide (NO) and cGMP signalling [55], and increased cellular lipofuscin accumulation [56].
Although the mechanism by which these age-related defects occur remains uncertain, there is evidence that changes in the redox balance within the Leydig cells are involved. In steroidogenic cells, ROS production would be expected to be particularly high because in addition to the mitochondrial electron transport chain, steroid hydroxylation by the cytochrome P450 enzymes produce ROS [57, 58]. In rat elderly LCs, it has been demonstrated that antioxidant defence molecules are reduced (superoxide dismutase-1 and -2, glutathione peroxidase, and glutathione) and the superoxide content is significantly increased compared to young rat LCs [59, 60]. Also, lipid peroxidation was found increased as a consequence of changes in the redox balance [59].
This may have significance for LC function because of the detrimental effects that ROS can have on critical components of the steroidogenic pathway [61-64].

6.2. Studies on mice

In contrast with studies on rats, recent studies on mice demonstrated how the different populations of cells in the testis are strictly correlated. Rebourcet D. et al (2017) [65] demonstrated that at all ages the size of the SC population is predictive of resulting cell composition. A reduction in SC number/proliferation at any age will therefore lead to a proportional decrease in LC and germ cell numbers. Moreover, Curley M. et al (2018) [66] showed how in a model of premature aging, intrinsic aging of LC population alone is not enough to cause T serum deficiency. Thus, they conclude that age-related LC dysfunction is largely driven by aging of the supporting testicular microenvironment.

AIMS

The decline in serum Testosterone concentration with aging in men has been demonstrated in several papers. All that is known about the mechanisms underlying this decline, derives from both *in vivo* and *in vitro* studies in rodent primary LCs and cell lines. Very few information about LC aging in men are available, and most of them are mainly of morphological nature.

The scarce knowledge about those mechanisms is due to the difficulty of obtaining biopsies from adult healthy men and to the lack of a suitable experimental model.

Thanks to an important collaboration with the Dipartment of General Surgery “Paride Stefanini” at the University of Rome (La Sapienza), our laboratory was allowed to obtain biopsies of testicular parenchyma from heart beating organ donors of various ages. In addition, thanks to a collaboration with the Department of Growth and Reproduction (Rigshospitalet, Copenhagen, Denmark) 24 more patients referred to the andrology clinic with normal spermatogenesis and hormone levels were included in this study.

Moreover, in our laboratory an *in vitro* organ culture model was recently developed.

The main objective of this study has been to elucidate the changes happening in human testicular morphology and function, with a particular focus on LCs, during aging.

Our working hypothesis was that T decline observed in serum was the result of both a reduction of LC number and a reduction in their functionality.

To address this hypothesis my PhD project plan has been divided as follows:

1: To investigate morphological differences through a histological analysis of testicular parenchyma of young and elderly donors;

2: To evaluate if there were changes in SC and LC number with aging;

3: To evaluate the expression at gene level of steroidogenic pathway enzymes and other markers of LC functionality;

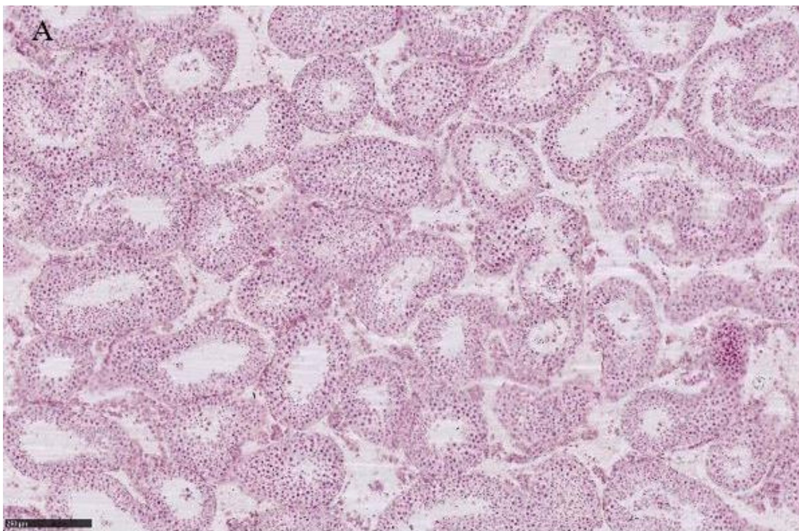
4: To assess the ability of producing androgens by *in vitro* cultured tissues both in basal conditions and under stimulation by gonadotropins.

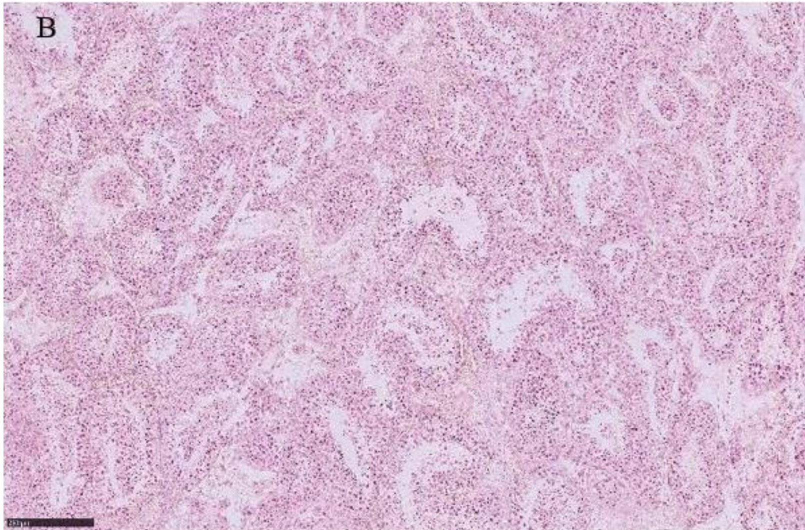
RESULTS

1. Changes in testicular morphology during aging

1.1. Histological analysis of testis

Firstly, we evaluated the morphology of the testicular parenchyma in young and elderly donors. Sections taken from different areas of the testis of each donor were stained with Carmalum and observed by light microscopy.
All samples showed normal spermatogenesis with both tubular and interstitial compartments well represented.
Testicular parenchyma of young donors (19-49-year-old) was found compact and with tubules full of germ cells at different stages of differentiation as shown in **figure 9A, B, C, D,** in which representative photographs of testis sections from 4 different young donors are reported.

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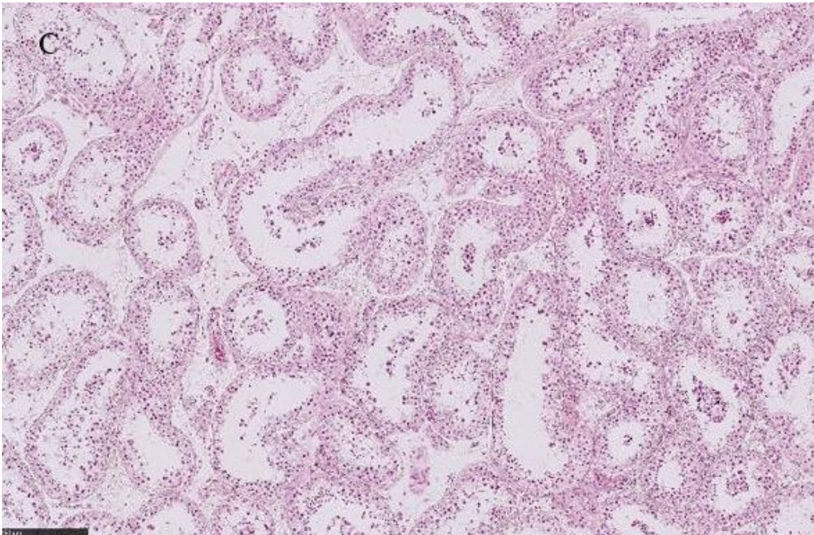
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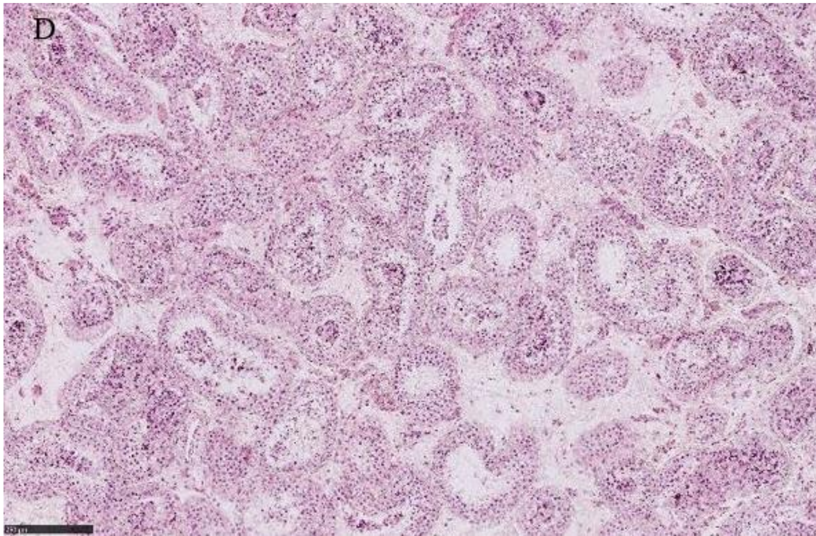
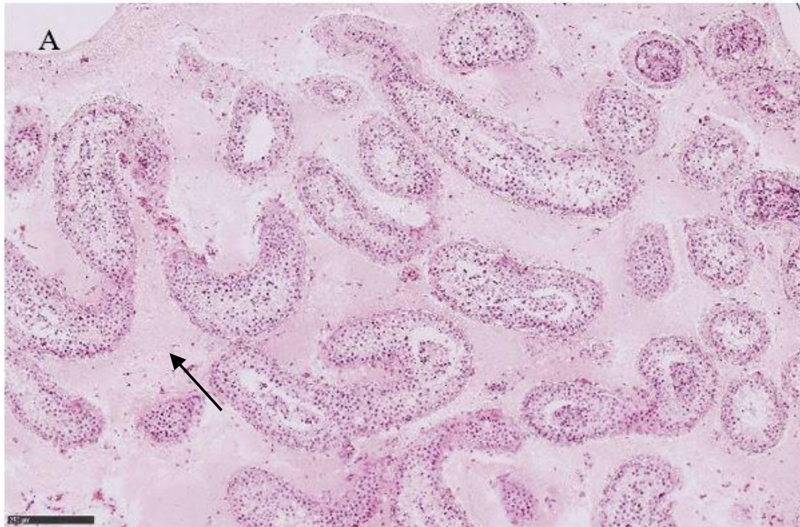


***Figure 9** Morphological analysis of testis from: **A,** donor #9, 19-year-old; **B,** donor #19, 19-year-old; **C,** donor #30, 35-year-old; **D,** donor #24, 41-year-old. Scale bars: 250 µm.*

Testicular parenchyma of elderly donors (50-85-year-old) was found with less seminiferous tubules, a reduction of tubules full of germ cells (empty tubules or sclerotic tubules) and also with an increased amount of fibrotic tissue in the interstitium as shown in **figure 10A, B, C, D**. Another typical characteristic of elderly donors was the thickening of the peritubulum.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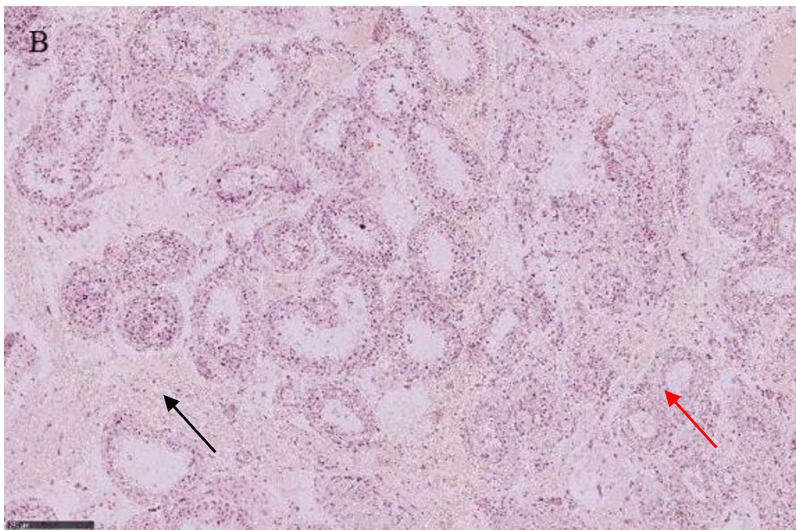
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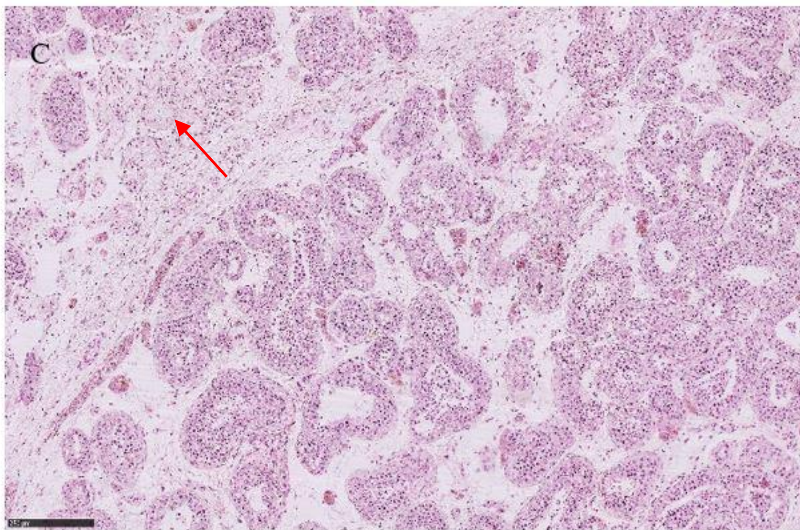


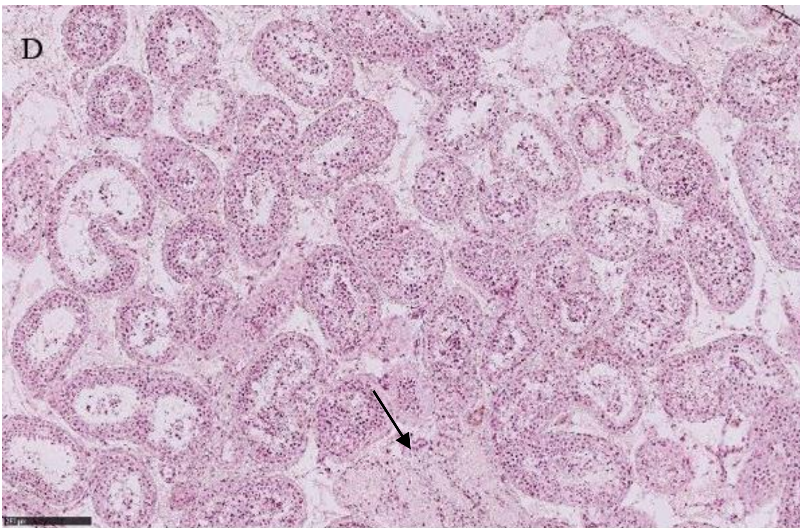
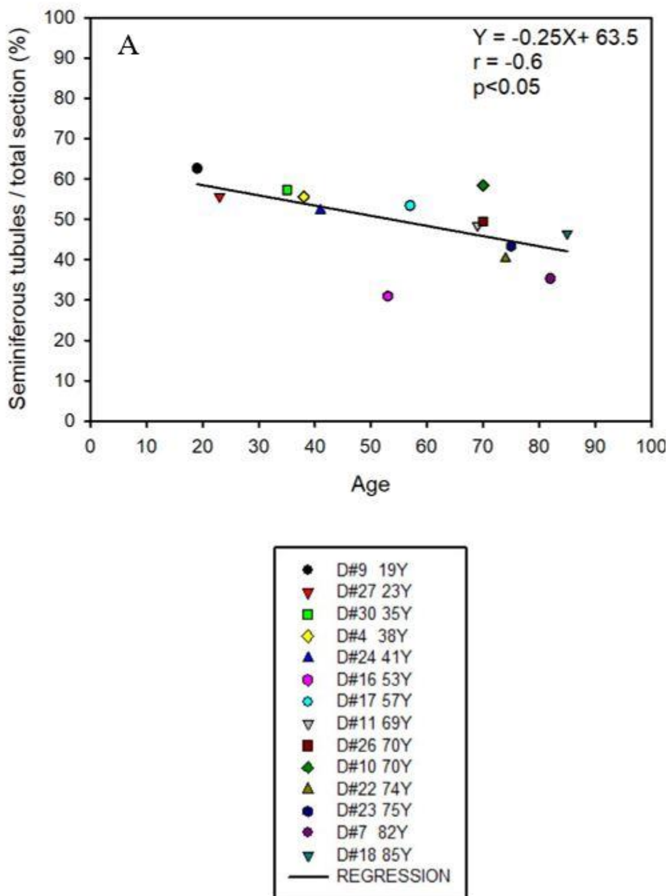


Figure 10 *Morphological analysis of testis from:* ***A,*** *donor #11, 69-year-old;* ***B,*** *donor #22, 74-year-old;* ***C,*** *donor #23, 75-year-old;* ***D,*** *donor #18, 85-year-old. Scale bars: 250 µm. Red arrows indicate empty or sclerotic tubules. Black arrows indicate fibrotic tissue.*

1.2. Quantification of testis morphological changes observed at the microscopic examination

To quantify the morphological alterations observed on testicular sections of the donors, we measured the total area of a histological section and the area occupied by seminiferous tubules using NDP view 2 software. The area occupied by interstitial compartment was calculated subtracting the area of the seminiferous tubules from the total area. We analyzed 4 sections randomly selected in different regions of the testicular parenchyma in 14 donors (5 young and 9 elderly donors).



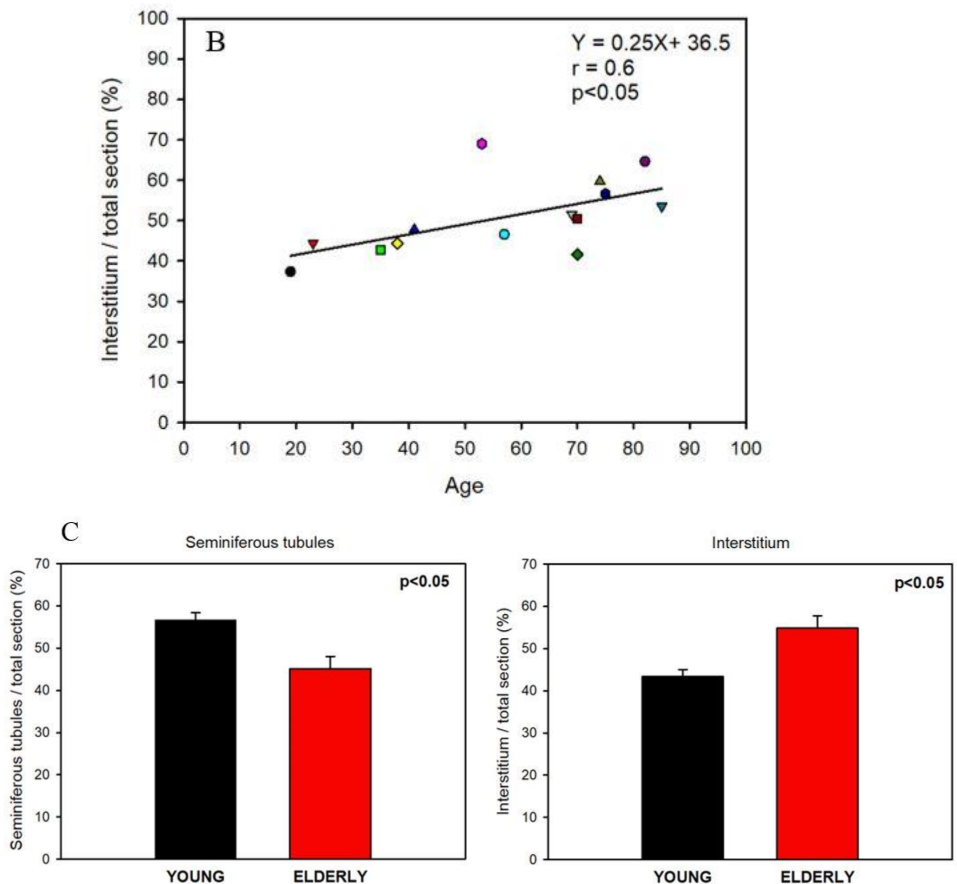


Figure 11A, B *Correlation analysis between age and percentage of the area occupied by seminiferous tubules or interstitium. Donors analysed are reported in the insert. Pearson correlation test was performed. In the graphs the correlation coefficients r and the p values together with the regression line equations are shown.* ***C*** *Comparison between young and elderly groups (young 19-49-year-old and elderly 50-85-year-old). T-test was performed.*

We evidenced a significative negative correlation between age and the area of the parenchyma occupied by seminiferous tubules and a significative positive correlation between age and the area of the parenchyma occupied by interstitium (Fig 11A, B).
The same data were grouped to compare the morphological changes between young and elderly donors. Figure 11C shows that there was

a significant increase in the interstitial area, accompanied by a decrease of the seminiferous tubule area, in the elderly compared to the young group.

2. Changes in Leydig and Sertoli cell number during aging

2.1. Leydig and Sertoli cell counting

Whether the number of LCs changes during aging is still a controversial issue, while the decrease in Sertoli and germ cell number is generally accepted [41-43] [67].
We evaluated LC and SC number in our samples performing an immunohistochemical (IHC) analysis of testicular sections using specific markers. We selected SOX9, which is a transcription factor that plays a pivotal role in male sexual development and is specifically expressed by SCs [68]. For LC staining, we selected two different markers, CYP17A1 and 3β-HSD, which are enzymes of the steroidogenic pathway. A similar number of cells was counted when LCs were immunoassayed with CYP17A1 or 3β-HSD antibodies in the same sample.
We performed two independent IHC experiments on sections taken from different areas of testicular parenchyma for each donor.
3βHSD+, (or Cyp17A1+) and SOX9+ cells were counted in 20 fields of 0.28mm² area under the light microscope or scans analysed using NDP view 2 software.
Interestingly, the correlation analysis revealed a significative decrease of both the number of LCs and SCs with age as shown in **figure 12A, B.**

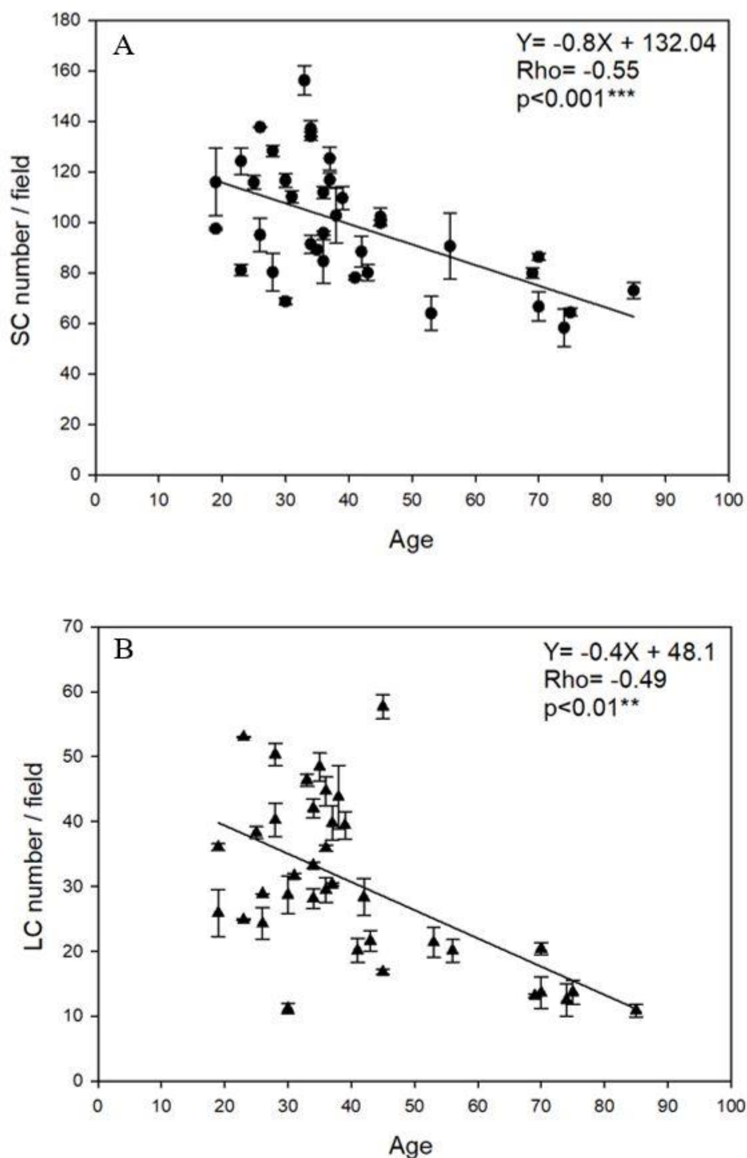


Figure 12 *Correlation analysis between age and* ***A*** *SC number,* ***B*** *LC number. Spearman correlation test was performed. In the graphs the correlation coefficient rho and the p values together with the regression line equations are shown.*

Our observation of aging impact on both Sertoli cell and Leydig cell numbers prompted us to identify a direct relationship between these two cell populations in each donor /subject testis. Fig. 13 shows that there was a highly significant positive correlation (rho=0.57; p<0.001) between SC number and LC number, indicating that in humans, at all ages, the size of one of these two cell populations correlates with that of the other one. These data are consistent with those recently published by Rebourcet et al (2017) [65] demonstrating that in a cell ablation mouse model the SC number defines and predicts LC population in the adult mouse testis.

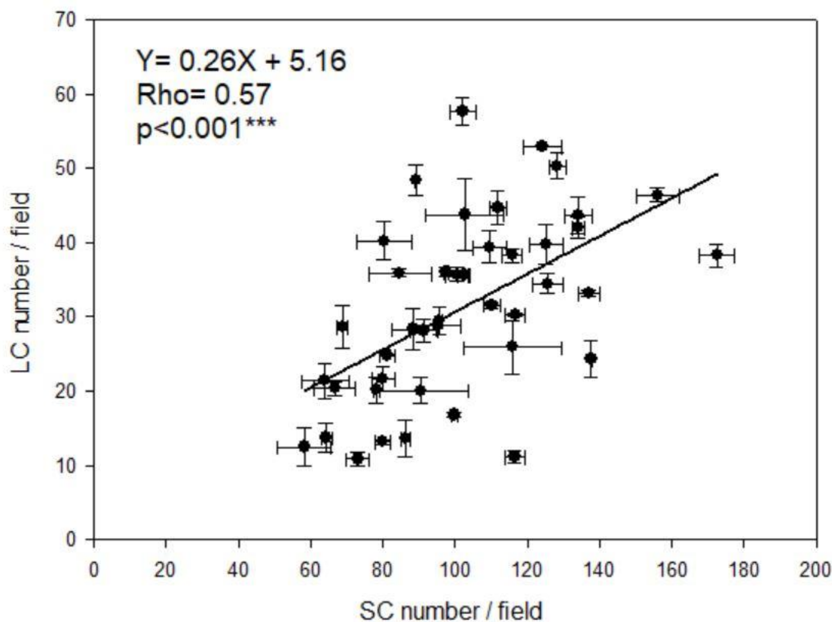


Figure 13 *Correlation analysis between SC number and LC number. Spearman correlation test was performed. In the graphs the correlation coefficient rho and the p value together with the regression line equation are shown.*

3. Leydig cell micronodules distribution analysis

To better investigate LC pattern we evaluated LC micronodules distribution in our samples. Micronodules are group of 15 or more LCs and are a common finding in testicular biopsies from men with

different disorders of spermatogenesis. Interestingly, they are associated with low T/LH ratio [14]. Therefore we hypothesized that morphological alterations of testis interstitial compartment in aged men could be accompanied by an increased number of LC micronodules.
As for the quantification of morphological changes, we measured the total area of at least 5 sections for each donor visualized with NDP view 2 software. Then we quantified the area occupied by micronodules in each section and the results are reported as percentage of the total area.
We carried out a correlation analysis between area occupied by micronodules and age. As shown in **figure 14**, the 2 parameters were found to be not correlated.

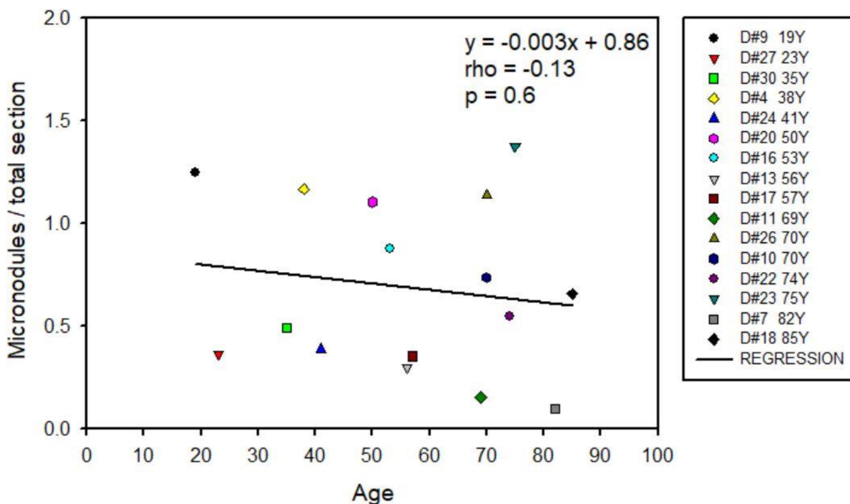


Figure 14 *Correlation analysis between area occupied by micronodules (percentage of total area of the section) and age. Donors analysed are reported in the insert. Spearman correlation test was performed. In the graph the correlation coefficient rho, the p value together with the regression line equation are shown.*

Moreover, we classified LC micronodules in three classes on the basis of their size (number of LCs/micronodule): 15-19 LCs, 20-30 LCs and $\geq$ 30 LCs/cluster. The number of micronodules for each class was counted in at least five testis sections from both young

and elderly donors. When we compared the frequency of the three classes of micronodule size in the two age groups, no significant differences were found (Figure 15).

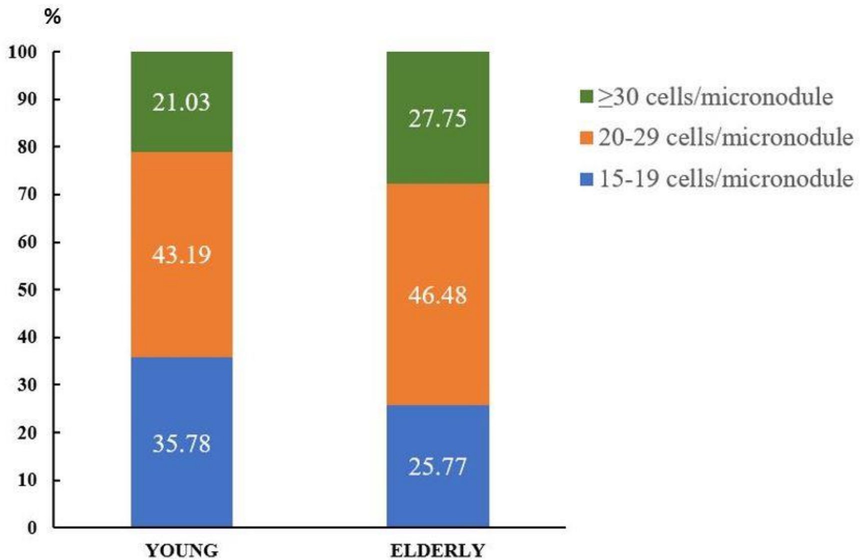


Figure 15 *Frequency of the three classes of LC micronodules in young and elderly donors. Chi-square test was performed. P>0.05.*

4. Gene expression analysis

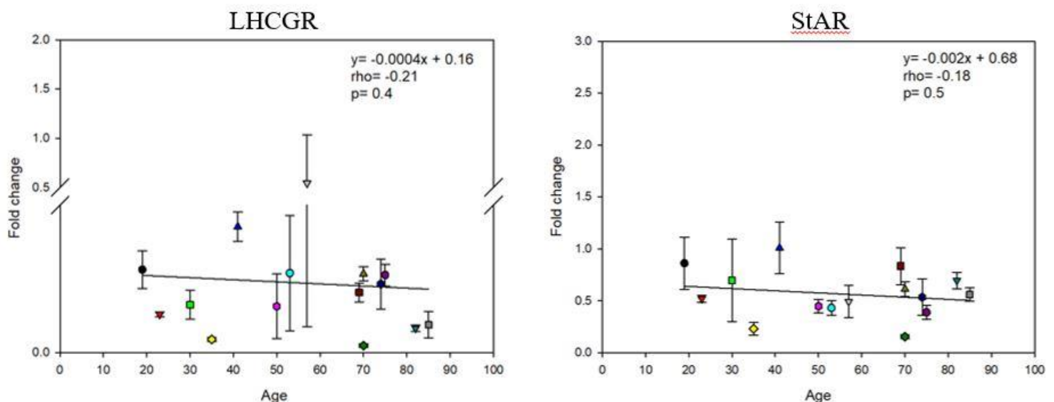
4.1. Setting the experiments

The next step was to evaluate if there were changes in LC functionality with aging. We started from a gene expression analysis of the steroidogenic pathway enzymes. First, we tested a panel of genes identified by Svingen T et al (2014) [69] as the most stable endogenous normalizing genes for expression analysis in adult human testis, in particular ribosomal protein S20 (RPS20), RPS29 and GAPDH genes. We found that both RPS29 and GAPDH expression had an acceptable degree of variability among our donors (data not shown), thus we chose RPS29 as the housekeeping gene.

In addition, we arbitrarily decided to use donor #20, 50-year-old, as the calibrator for the qRT-PCR analysis, because this donor had an intermediate age between young and elderly donors.

4.2. Analysis of steroidogenic pathway enzymes and other LC markers expression

We evaluated by qRT-PCR analysis the expression of the following genes: LHCGR, StAR, CYP11A1, CYP17A1, 3β-HSD2, 17β-HSD3, and we analysed the relationship between each gene expression and the donor age. The statistical analysis revealed no significant correlation for any of the genes tested; however LHCGR (rho= -0.21, P= 0.4), StAR (rho= -0.018, P= 0.5), CYP11A1 (rho= -0.03, P= 0.9) and 17β-HSD3 (rho=-0.48, P=0.08) showed a negative trend, whereas CYP17A1 (rho= 0.3,P= 0.4) and 3β-HSD2 (rho= 0.25, P=0.3) showed a positive trend with age (Fig. 16 and Table 1).



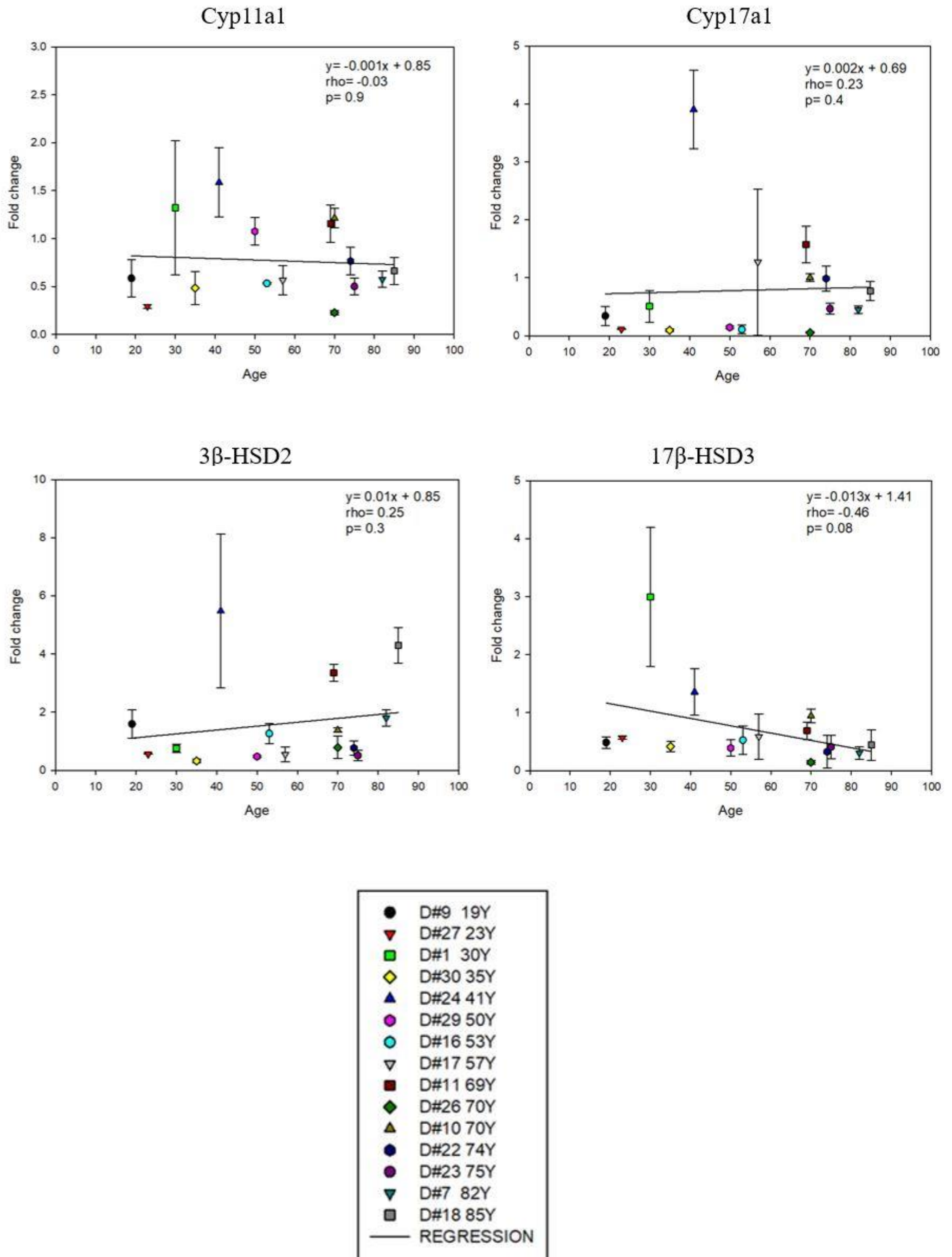

Cyp11a1
y= -0.001x + 0.85
rho= -0.03
p= 0.9
Fold change
Age
Cyp17a1
y= 0.002x + 0.69
rho= 0.23
p= 0.4
Fold change
Age
3β-HSD2
y= 0.01x + 0.85
rho= 0.25
p= 0.3
Fold change
Age
17β-HSD3
y= -0.013x + 1.41
rho= -0.46
p= 0.08
Fold change
Age
D#9 19Y
D#27 23Y
D#1 30Y
D#30 35Y
D#24 41Y
D#29 50Y
D#16 53Y
D#17 57Y
D#11 69Y
D#26 70Y
D#10 70Y
D#22 74Y
D#23 75Y
D#7 82Y
D#18 85Y
REGRESSION

Figure 16 *Correlation analysis between the expression of the gene indicated on the top of each graph and age. Donors analyzed are reported in the insert. Spearman correlation test was performed. In the graph, the correlation coefficients rho, the p values together with the regression line equations are shown.*

Given that the steroidogenic pathway gene expression did not change significantly in aged men, we asked the question whether the expression of INSL3 decreased during aging. Indeed, this testicular hormone is known to reflect the LC functional status and its serum levels decline with age in men [7]. Results in fig. 17 show that INSL3 did correlate with age in a significant negative manner (rho= -0.52, P<0.05). Importantly, the decline in INSL3 gene expression in aged men agrees with our finding of age-related LC number decrease.

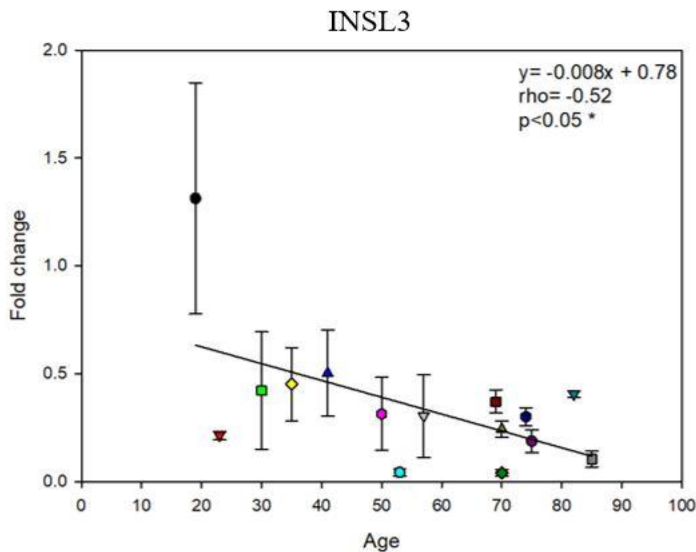


Figure 17 *Correlation analysis between the expression of INSL3 gene and age. Donors analyzed are the same reported in the insert of figure 6. Spearman correlation test was performed. In the graph, the correlation coefficients rho, the p values together with the regression line equations are shown.*

A summary of correlation coefficients and p values for each gene evaluated is reported in the **table 1**.

Gene	Rho	P value
StAR	-0.18	0.5
CYP11A1	-0.03	0.9
CYP17A1	0.23	0.4
3β-HSD2	0.25	0.3
17β-HSD3	-0.46	0.08
LHCGR	-0.21	0.4
INSL3	-0.52	P<0.05 *

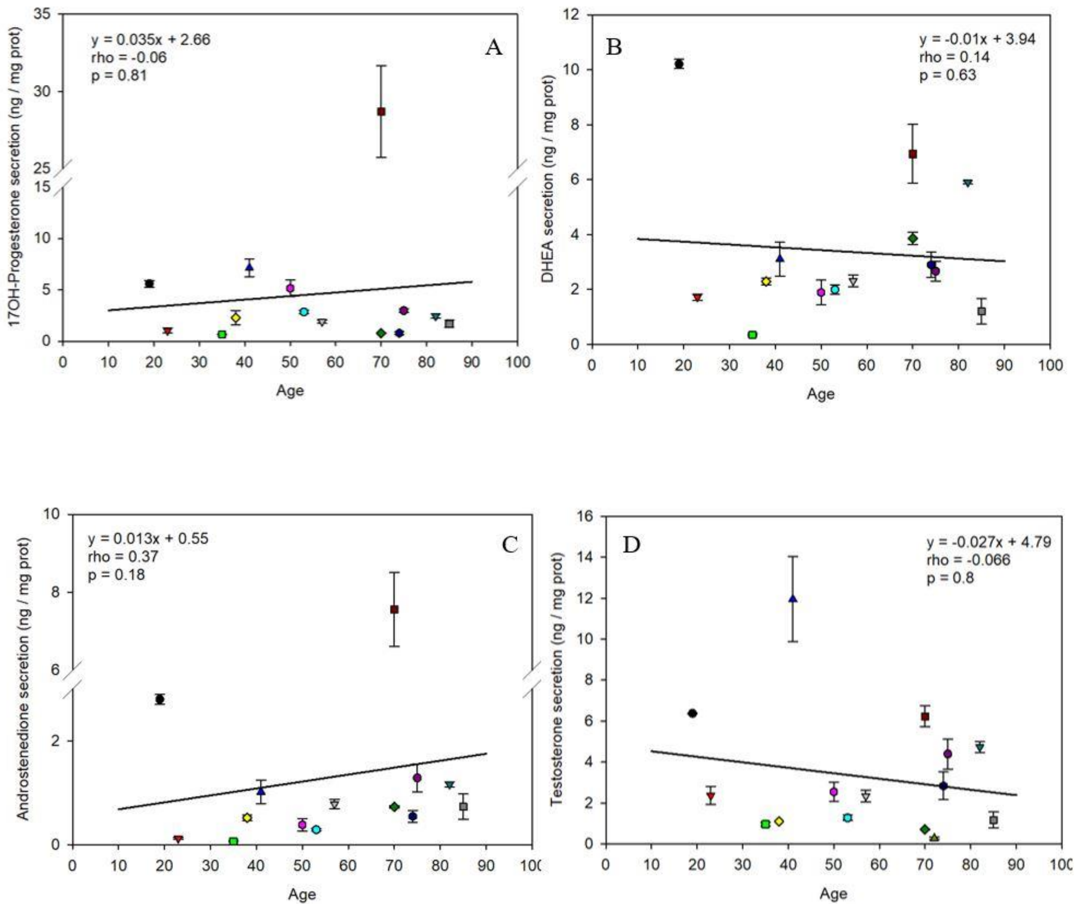
Table 1 *Summary of the rho coefficients and p values of the correlation analysis between each gene expression and age.*

5. Analysis of androgen production by in vitro cultured human testis fragments from donors of different ages

5.1. Androgen secretion in basal conditions

Because normal human adult testicular tissue is difficult to obtain, we routinely cryopreserved small fragments of testis from each organ donor obtainable. With the aim to investigate T, Androstenedione (A), Dehydroepiandrosterone (DHEA) and 17-Hydroxyprogesterone (17OHP) *in vitro* production and the effect of age, we used an organ culture method recently set up in our laboratory, which allows to maintain a good preservation of the testis tissue in culture for short times. We therefore incubated cryopreserved testis fragments from donors of different ages (5 young and 10 elderly) for 3h in basal conditions and androgens were measured in the culture media. It is of interest that the four androgens secreted were simultaneously measurable by mass spectrometry in all the donors chosen for the analysis, yet displaying a great interindividual variation. The concentrations of 17-OH-P, DHEA and T, normalized per amount of tissue, were similar and two times higher than that of A. To determine whether the four androgen levels correlated with the donor age, we performed a Spearman correlation analysis. Figure 18 shows the

results. None of the androgens significantly correlated with age, however 17-OH-P and T showed a weak negative trend, whereas DHEA and A showed a positive one.
These data indicate that the cryopreservation of human testis biopsies does not alter the morphological integrity of the tissue and allows to maintain it in culture for short period of times. Interestingly, we did not observe a significant age-related change in the androgen production.



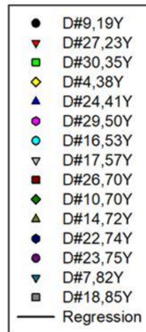


Figure 18 *Correlation analysis between androgen secreted by cryopreserved tissue cultures in basal conditions and age.* ***A*** *17-Hydroxyprogesterone* ***B*** *DHEA,* ***C*** *Androstenedione and* ***D*** *Testosterone. Donors analyzed are reported in the insert. Spearman correlation test was performed. In the graph, the correlation coefficients rho, the p values together with the regression line equations are shown.*

We then investigated the in vitro steroidogenic ability of testis from organ donors of different ages using fresh tissue. We therefore cultured testis fragments from 10 donors (3 young and 7 elderly) immediately after gonad surgical removal and collection. Similarly, to the four androgens secreted after cryopreservation, those secreted into the media after 3h by fragments cultured before cryopreservation were measurable by mass spectrometry in all the donors chosen for the analysis, yet displaying a great interindividual variation. Interestingly, the concentrations of 17 OH-P and T, normalized per amount of tissue, were approximately 10 times higher than those of A and DHEA. To determine whether the four androgen levels correlated with the age, we performed a Spearman correlation analysis. Table 2 reports the correlation coefficients rho and the P values for each steroid. A weak negative trend was found for 17-OH-P, DHEA and T, whereas a positive one for A. However, none of the androgens significantly correlated with age.
These data indicate that the lack of correlation between androgen secreted by LC after testis cryopreservation and donor age (Fig.18) is not due to the freezing of testis biopsies, because similar results were obtained using fresh tissue.

Androgen	Rho	P value
17OHP	-0,09	0,8
DHEA	-0,07	0,8
A	0,23	0,5
T	-0,01	0,9

Tables 2 *Summary of the rho coefficients and p values of the correlation analysis between androgen secreted in the media by fresh organ cultures and age.*

5.2 Differences in androgen secreted by fresh and cryopreserved testis fragments

To gain more insight into the possible differences in androgen production by LCs before and after the cryopreservation of the tissue, testicular fragments from various donors of different ages were cultured *in vitro* for 3h in basal conditions, immediately after collection and after the cryopreservation. Four androgens secreted into the culture medium were measured: T, A, DHEA and 17-OH progesterone. The concentration of the androgens was normalized per mg of tissue, and the average of the fresh samples and the frozen ones from the same donors (#16, #17, #22, #23, #24, #26, #27, #29, #30) is shown in figure 19. Comparing the data obtained from the fresh and the cryopreserved tissues, we observed that T levels significantly decreased after cryopreservation, while A and DHEA levels significantly increased. The 17-OH-P increment was found not significant. The four androgen profiles were observed in all donors analysed regardless of age. These data suggest that cryopreservation could cause changes in specific enzymatic activities of testicular steroidogenesis such as P450c17, 3βHSD2 and 17βHSD3.

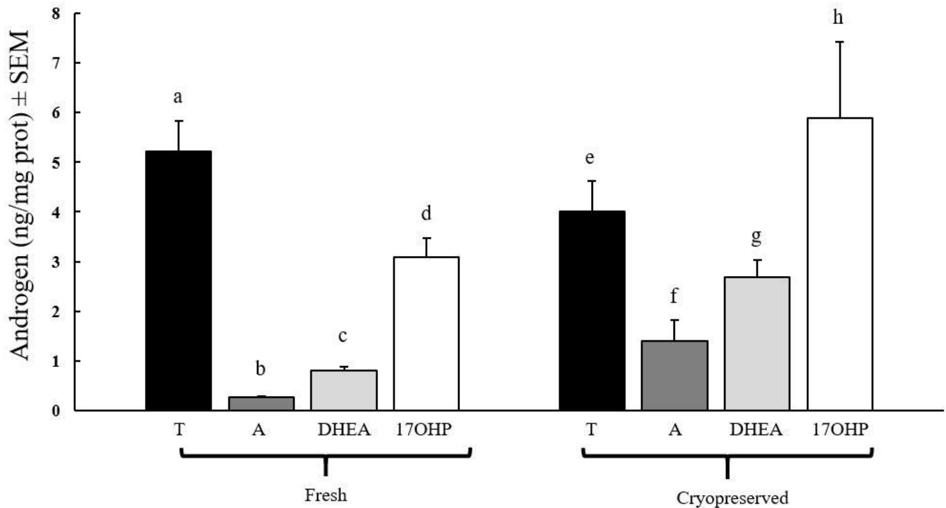


Figure 19. *Comparison of four androgen levels secreted in basal conditions by LC in fresh and cryopreserved testicular tissue obtained from different donors of various ages. The graph shows that T production declined after cryopreservation whereas A, DHEA and 17-OH-progesterone increased. Data are presented as means ±SEM. Mann Whitney Test was performed. a vs e: p<0.05, b vs f: p<0.001, c vs g: p<0.001, d vs h: not significant. n=9.*

5.3 Analysis of age-dependent testosterone and 17hydroxyprogesterone secretion by LCs in response to gonadotropin stimulation

Next, we evaluated the in vitro LC response to gonadotropin in terms of production of testosterone, the main and final product of testicular steroidogenesis, and 17-OH-progesterone, an early steroid of the pathway. We cultured for three hours testis fragments obtained immediately after collection from 4 young (#19, #27, #30, #24) and 6 elderly (#20, #29, #16, #17, #22, #23) donors, in the presence or absence of 100 nM rh-LH or 100 nM rh-CG. Fig. 20 shows the results of the response in terms of T. LH did not cause a significant stimulation of T compared to control samples in all donors examined except for donor #16, whereas hCG was more potent, significantly stimulating T secretion by testis fragments

from donors #16, #17, #20, #27. Fig. 21 shows the results of the response in terms of 17-OH-P. This steroid was significantly stimulated by LH in two donors (#29, #17), whereas significantly increased after hCG treatment in five donors (#16, #17, #19, #20, #23).

These data demonstrate that: a) the response to gonadotropin was highly variable among all donors; b) the response was not age-dependent; c) hCG was more potent than LH in eliciting an increase of steroid production; d) 17-OH-P was better stimulated (seven responders) by both gonadotropins compared to T (five responders).

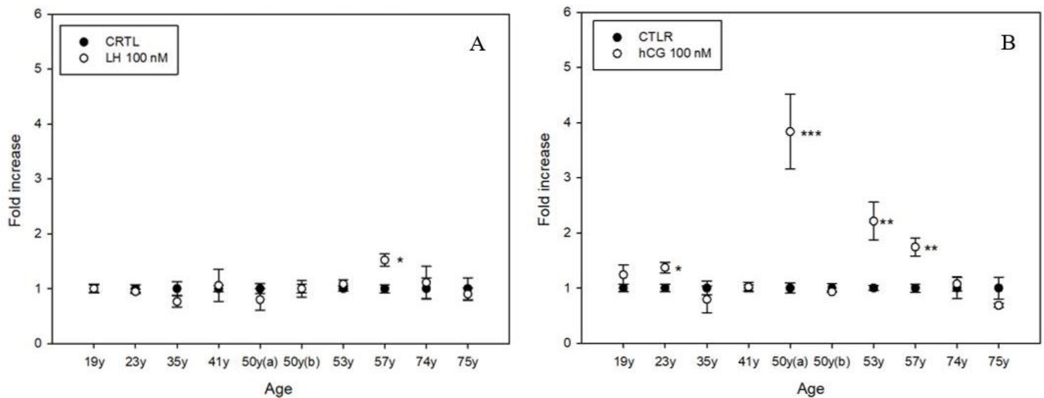


Figure 20 *LC response to rh-LH (A) and rhCG (B) in terms of T production, using fresh testicular fragments. Data are expressed as fold increase compared to control. T-test was performed. * p< 0.05; ** p<0.01, ***p<0.001.*

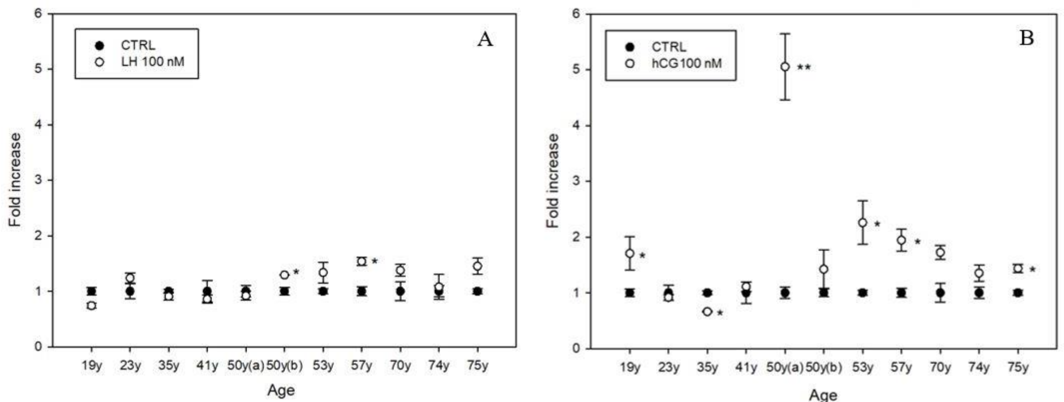


Figure 21 *LC response to rh-LH (A) and rhCG (B) in terms of 17-OH progesterone production, using fresh testicular fragments. Data are expressed as fold increase compared to control. T-test was performed. * p< 0.05.*

We then asked the question whether androgens secreted by LCs could be stimulated by gonadotropin after cryopreservation of testicular tissue. We cultured for three hours cryopreserved testis fragments from 3 young (#9, #27, #24) and 5 elderly (#16, #10, #22, #23, #18) donors and we measured T levels secreted into the media in the presence or absence of 100 nM rh-LH or 100 nM rh-CG. Results are shown in Fig. 22. Testosterone significantly increased after LH stimulation only in donor #22, and after hCG stimulation in donor #18. No response was observed in all the other donors. These data agree with the finding that testosterone levels were reduced after cryopreservation and demonstrate that freezing testicular tissue causes an impairment of LC response to gonadotropins.

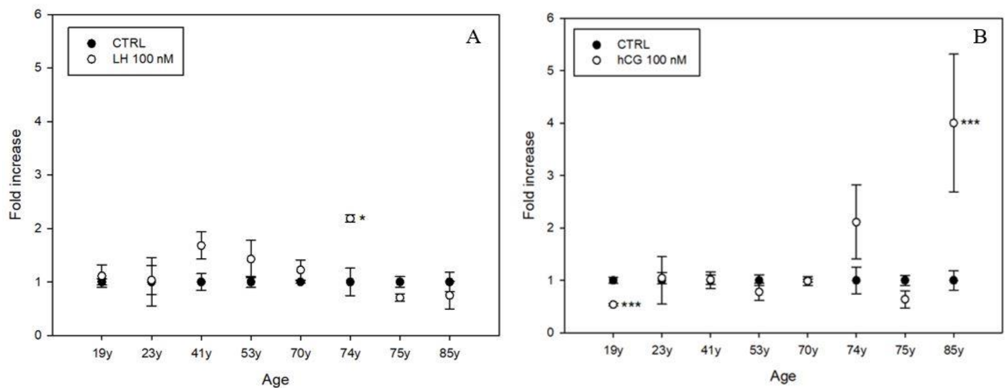


Figure 22 *LC response to 100 nM rh-LH (A) and rhCG (B) in terms of T production, using cryopreserved testicular fragments. Data are expressed as fold increase compared to control. T-test was performed. * p< 0.05; ***p<0.001.*

5.4 Changes in LC gene expression in response to gonadotropin stimulation

In order to determine if LC gene expression profile changed after gonadotropin stimulation, we *in vitro* cultured for three hours testis fragments from two donors (#27, 23-year-old and #23, 75-year-old), in the presence or absence of 100 nM rh-LH and rhCG, both before and after cryopreservation. The steroidogenic enzymes, LHCGR and INSL3 gene expression was evaluated by qRT-PCR and expressed as fold change related to unstimulated control. In contrast to the finding that T and 17-OH-P were significantly stimulated by hCG, when fresh testis fragments were used from the two donors, respectively, the expression of LC specific genes did not change under gonadotropin stimulation. Similarly, no effect of the gonadotropins was observed when cryopreserved testis fragments from the same donors were analysed (data not shown).

DISCUSSION

Androgen production under the strict endocrine control of the pituitary gland via LH secretion is one of two main testicular functions. Testosterone is the most important androgen secreted and it acts on several different districts of the body and also on the LH production by the pituitary gland, as a negative feedback.
In humans, aging is associated with a decline in serum Testosterone concentration (late onset hypogonadism (LOH)) due to a primary insufficiency of testis function. This deficiency is accompanied by several metabolic disorders like infertility, loss of muscles mass, obesity, bone fragility which can be grouped together under the name of metabolic syndrome. Hypogonadism is believed to worsen the metabolic status of the body, generating a vicious circle resulting in increased morbidity and mortality. Environmental factors, including pollutants, and lifestyle (sedentariness, wrong eating habits, smoke) have an impact on testicular function, resulting both in reduced Leydig cell function and infertility as well as in permanent epigenetic modifications of germ cells transmitted to the progeny.
While some of the defects leading to androgen deficiency have been demonstrated in rodents [36], the mechanisms by which LC testosterone production becomes compromised in aged men remains to be established.
The general aim of this project has been to investigate the impact of age on human testis, focusing mainly on Leydig cell function, using biopsies from heart beating organ donors of different ages. Although the number of donors available was limited and their serum gonadotropin and androgen concentrations were unobtainable, it is worth noting that our study is the first one carried out on healthy men without reproductive abnormalities or testicular infections, selected for organ (heart, liver, kidney) donation and the causes of death of whom were mainly haemorrhagic, stroke and accident.
It is well known that aging is a deterioration process at the systemic level, compromising each district of the body. Thus, it is not surprising that also in the male gonad we were able to observe

morphological alterations of the tissue, consisting of a significant loss of the area occupied by seminiferous tubules, accompanied by an increased interstitial area, in the testicular parenchyma of elderly donors compared to young ones. Consistent with our finding, an age-related reduction of total volume of seminiferous tubules associated with an increase thickening of lamina propria have been previously observed in elderly men [67, 70, 71].
Age-related decrease in the number of germ cells, mainly spermatids, were also found in elderly men [42, 71, 72], and it is well established that semen parameters change with age, including semen volume, total sperm count, sperm percentage progressive motility, normal morphology. Unexpectedly, sperm concentration does not decline with increasing male age [73]. As for the influence of age on the number of Sertoli cells and Leydig cells this issue is rather debated. In this study we performed the counting of SC and LC numbers based on their specific identification on testis sections immunoassayed for Sox9 and 3βHSD2/CYP17A1, respectively, from 37 subjects ranging in age from 19 to 85 years (24 with obstructive azoospermia and 13 heart beating organ donors). Interestingly, we found a significant negative correlation for both SC and LC number with age. Our finding that human aging is associated with a significant decline of SC number is in agreement with earlier studies which quantified SC population using only morphological criteria for cell identification, and reported that the number of SCs in the aged men was significantly lower than that in the young ones [43, 67, 71, 72]. In line with that, a marked reduction in the total number of SCs per testis was found in 30-month-old Brown-Norway rat [74], which is a strain unusually long-lived, representing a good model for studying age associated testis changes.
As for LC population change in aging men, conflicting claims characterize the small number of studies on this issue. Our observation that aging negatively affects LC number is consistent with most studies in humans. Indeed, a reduction in both LC number and other interstitial cells with advancing age was reported [41, 42, 71, 75]. In contrast, Petersen PM, et al. (2015) [43] did not found a decline in the LC number with age. This discrepancy may be because there is considerable variation in LC number between

individuals and because the tested subject number is relatively small in that study. In contrast, in the old Brown Norway rat, the age-associated diminished testosterone secretion could not be explained by a reduction in LC number [74, 76].

Of great interest, our results demonstrate a strongly positive significant correlation between SC and LC numbers, in each subject, at all ages. Recently Rebourcet D et al. (2017) [65] reported similar results in mouse, using a system to specifically ablate SCs. They demonstrated that the attrition of SC number at different points of mouse development leads also to a reduction in LC and germ cells numbers in the adult, pointing to the concept that SC number defines and predicts germ and LC population sizes. Another recent study from Curley M et al. (2018) [66] demonstrated that in a mouse model of premature aging, both SC and LC populations were reduced in number and also steroidogenesis was compromised. Surprisingly, when they selectively induced the premature aging in either LC or SC, they did not observe the same alterations, pointing to the idea that age-related disruption of testicular microenvironment and/or wider endocrine milieu together with LC intrinsic aging may cause the age-associated LC dysfunction.

Following the morphological analysis of human aging testis, we evaluated LC functionality in young and elderly group of organ donors. Of great interest, we found a significant decline in the expression of INSL3 in aging men, whereas *in vitro* androgen (T, A, DHEA, 17OHP) secretion did not correlate with age, although with a weak negative trend. INSL3 and testosterone are two specific markers of LC function, but are regulated very differently, being INSL3 insensitive to acute gonadotropin stimulation and constitutively expressed as a function only of the differentiation status and/ or the number of LCs [77]. It is interesting to note that we observed here a significant reduction of LC number associated with a lower expression of INSL3 in elderly men compared to young ones. Consistent with that, circulating INSL3 concentrations in humans have been reported to be reduced in advancing age [78] and in conditions associated with LC impairment [79]. The expression of INSL3 gene was found decreased at both RNA and protein level also in aging rat testis [80].

In marked contrast with INSL3, testosterone is highly variable within individuals and is acutely responsive to fluctuations in the hypothalamic–pituitary–gonadal axis. Although in men, as in rodents, aging is associated with lower serum testosterone concentrations, our data do not confirm that the LC steroidogenic function is affected by the advancing age. Both the basal gene expression of steroidogenic pathway enzymes and the levels of four androgens measured in the media after three hours in vitro cultures of testis fragments from organ donors did not decline with age. In addition, although the presence of LC micronodules is considered a histological sign of testicular androgenic failure, associated with morphological alterations of testis compartments, the frequency of these large LC clusters was not higher in elderly men. Taken together, these data suggest that the steroidogenic machinery of LCs remains unchanged as a man age. However, the small number of subjects in this part of the study and the short duration of *in vitro* culture may impact on our findings. Moreover, the donor serum hormone levels in vivo were not assessed. Indeed, gene expression can be influenced by several factors such as gonadotropin stimulation and paracrine/autocrine signals. In accordance with our observations, 3β-HSD and CYP17A1 steroidogenic enzyme transcripts did not change in old Brown Norway rat testis, yet the enzyme activities were reduced compared to young animals [81]. A similar deficit in testis steroidogenic enzymes was reported in aging mice [82].

The *in vitro* experimental model used in this study presents some advantages: it requires a small amount of tissue and most importantly mimics the *in vivo* situation, as the cell to cell interactions and the structural integrity of both interstitial and seminiferous tubule compartment are preserved. This approach allowed to assess androgen secretion in both basal conditions and under stimulation by recombinant gonadotropins. When fresh testis fragments were stimulated with recombinant gonadotropins, hCG was more efficient than LH at the same concentration in stimulating a response in terms of 17OHP and T secretion. Being 17OHP an earlier metabolite in the steroidogenic pathway, a higher number of donors was found to be responsive to hCG compared to T production. Consistent with our observations, it has been recently

reported that rhLH and rhCG display divergences in their potencies, efficacies and kinetics. Indeed, rhCG was more active compared to rLH in terms of intracellular cAMP production in human granulosa cells, mouse primary LCs and mouse Leydig tumour cells (mLTC-1) [83-85].

Furthermore, our results demonstrate that, despite the cryopreservation determined a moderate germ cell degeneration, the interstitial compartment was well preserved, indicating that also cryopreserved tissues can be used for *in vitro* experiments. However, T levels secreted into the media by fragments cultured after cryopreservation significantly decreased in comparison to those by fragments cultured immediately after collection, suggesting that cryopreservation might affects steroidogenic enzymes activity. Consistent with T reduction, A and DHEA levels secreted into the media of cryopreserved tissue cultures significantly increased, pointing to a particular vulnerability of 3βHSD2 and 17βHSD3 enzymes which convert DHEA to androstenediol and androstenedione to T, respectively. Another evidence reinforcing the concept that cryopreservation affects metabolism of LCs, is given by our results showing the response to gonadotropins in terms of T secreted into the culture media by cryopreserved fragments organ cultures. While hCG was able to stimulate the *in vitro* secretion of T at 100nM dose when the biopsies were used immediately after collection, the stimulatory effect of the hormone was lost when the fragments were cultivated after cryopreservation.

Based on our observations of changes in testicular morphology, the significant reduction in both LC and SC number and the functional data obtained *in vitro,* we can conclude that there is a cellular senescence in the aging human testis which is not associated to the *in vitro* ability to produce androgens. Thus, our results support the idea that LC dysfunction is largely driven by aging of the testicular microenvironment rather than aging of LC population alone.

MATERIALS AND METHODS

1. Human testis samples

a) Testicular biopsies were obtained from heart-beating organ donors under a collaboration with the Organ Transplants Center - Division of General Surgery, Policlinico Umberto I hospital (Rome, Italy).

Donor	Age
DOT#01	30
DOT#04	38
DOT#07	82
DOT#09	19
DOT#10	70
DOT#11	69
DOT#13	56
DOT#16	53
DOT#17	57
DOT#18	85
DOT#20	50
DOT#22	74
DOT#23	75
DOT#24	41
DOT#26	70
DOT#27	23
DOT#29	50
DOT#30	35

Table-3 *Age of the donors, ranging from 19 to 85-year-old.*

For each donor, the free and informed consent of the family concerned, was obtained. The Ethical Committee of the hospital approved the use of human material according to national guidelines for organ donation as issued by the Italian Ministry of Public Health. Biopsies were collected as previously described [86] from different areas of the testis. After collection, the biopsies were

transported in ice-cold phosphate buffered saline (PBS) (Sigma-Aldrich) to the laboratory and processed within 1 h.

b) Testicular biopsies were obtained from patients referred to the andrology clinic at Department of Growth and Reproduction at Rigshospitalet, Copenhagen, Denmark. Samples included in this study were collected in accordance with the Helsinki Declaration following approval from the local medical research ethics committee (permit no. H-1-2012-007). Patients gave their written and oral consent prior to any tissue being used and biopsy samples were identified in the department's patient database with the inclusion criteria that assessment of their biopsy showed tubules with complete spermatogenesis and their hormone levels (testosterone, SHBG, inhibin B, estradiol, LH and FSH) were in the normal range.

2. Histological analysis of samples obtained from organ donors

Fragments of the biopsies were routinely fixed in Bouin's fixative or 10% buffered formalin solution (Sigma-Aldrich) and embedded in paraffin (Sigma-Aldrich). Some other fragments of the biopsies were left unfixed and used for short-term cultures. The remaining parts of the biopsies were cut into fragments of $0.5cm^3$ and some fragments were dry frozen at -80°C, while the other ones were cryopreserved in liquid nitrogen. For cryopreservation, the testis tissue was immerged in a medium containing DMSO (1.1M), DMEM (1X), Fetal bovine serum (20%) and sucrose (0.15M) in cryovials on dry ice for 15 minutes. The vials were then transferred to -80°C for 24-48h, and then in liquid nitrogen as previously described [87].
For morphological analysis, 5 μm-thick sections of samples fixed in Bouin were mounted on the Polysine slides (Thermo Fisher Scientific), stained with Carmalum solution composed by 1g of Carmic acid plus 10g of potassium aluminium sulfate (Merck-Millipore, Darmstadt, Germany) in 200ml of distilled water and analysed. All the samples included in this study showed well-preserved testicular parenchyma and a normal spermatogenesis.

Morphological analysis was performed using the optical microscope (Zeiss) and the images were acquired with the Zen Lite 2 software (Zeiss).

3. Quantification of morphological changes and micronodules distribution between young and elderly donors

Sections for histological analysis were scanned with NanoZoomer 2.0 HT (Hamamatsu Photonics, Herrsching am Ammersee, Germany). We quantified the area occupied by seminiferous tubules in 4 different sections taken in different areas of testicular parenchyma for each donor using NDP view version 1.2.36 (Hamamatsu Photonics), which is the specific software to analyse scans taken with NanoZoomer 2.0 (Hamamatsu Photonics, Herrsching am Ammersee, Germany). The area occupied by interstitium was obtained subtracting the area occupied by seminiferous tubules from the total area of a section. Data were expressed as percentage of the total area of a section.
For the evaluation of micronodules distribution, we quantified the area occupied by micronodules in at least 5 different sections taken in different areas of testicular parenchyma for each donor using NDP view version 1.2.36 (Hamamatsu Photonics). Data were expressed as percentage of the total area of a section.

4. Immunohistochemistry (IHC)

Immunohistochemistry experiments were performed using two different protocols, because samples kindly provided by the Dept. of Growth and Reproduction Rigshospitalet, Copenhagen, Denmark were fixed in a different fixative.
Protocol used for samples obtained from organ donors:
- 5 μm thick paraffin sections of samples fixed in 10% buffered formalin solution from human testis samples were randomly collected from different areas of testicular parenchyma of donors arrived at the DAHFMO department, Rome, and mounted on polylysine-coated slides (Thermo Fisher Scientific). Dewaxed and rehydrated sections were incubated in 1X Tris-EDTA citrate buffer (10mM, pH 7.8) (UCS Diagnostic, Rome, Italy) in a microwave

oven for antigen retrieval at 700W three times for 3 min. After quenching of endogenous peroxidase and blocking of nonspecific binding with Super Block (UltraTek HRP Anti-Polyvalent kit, ScyTek Laboratories), sections were incubated for 2h 30' at RT with a mix of 1:150 diluted primary polyclonal anti-3βHSD antibody (Santa Cruz**,** sc-30820) and 1:300 diluted primary monoclonal anti-SOX9 antibody (Cell Signaling**,** D8G8H). After washing, sections were processed using the avidin-biotin peroxidase complex (ABC) procedure, according to the manufacturer's instructions (UltraTek HRP Anti-Polyvalent kit, ScyTek Laboratories). Negative control was performed omitting the primary antibodies. Peroxidase activity was revealed using DAB (Roche Diagnostic, Basilea, Switzerland), and nuclei were shortly counterstained with Mayer Hematoxylin (Sigma-Aldrich). After washing, sections were dehydrated through the ascending ethanol series and permanently mounted with EUKIT (Kindler GmBH).

Protocol used for Dept. of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark samples:

- 5 µm thick paraffin sections of samples fixed in Modified Stieve fixative (GR fixative, composition: 200 mL 37% Formaldehyde, 40 mL acetic acid added to 1 L of 0.05 M phosphate buffer, pH 7.4 VWR, Bie & Berntsen, Copenhagen, Denmark). from human testis samples were randomly collected from different areas of testicular parenchyma of donors kindly furnished by the Dept. of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark, and mounted on Menzel-Gläzer SuperFrost slides (Thermo Scientific, #1510.1250). Dewaxed and rehydrated sections were incubated in TEG buffer (10 mMTris, 0.5 mM EGTA, pH 9.0) in a pressure cooker for antigen retrieval at 110°C for 30 minutes. After quenching of endogenous peroxidase with methanol LC-MS grade 99% and blocking of nonspecific binding with a blockade solution (20 ml horse serum IMMPRESS kit MP-7405 + 5g BSA + 80 ml TBS), sections were incubated over night with SOX9 antibody (Millipore AB5535) for the SC staining and with CYP17A1 antibody (Abcam ab134910) for the LC staining. The day after, slides were washed in TBS (Trisma base 6,06 g, NaCl 8,7 g, Demineralised water to 1 L, pH 7,4 From the Hospital pharmacy) and then incubated with the 2°ndary antibody (ImmPRESS HRP

reagent: Anti-Rabbit MP7401, Vector Laboratories). Negative control was performed omitting the primary antibodies. Peroxidase activity was revealed using ImmPACT DAB (Peroxidase substrate kit SK-4105, Vector Laboratories) and nuclei were shortly counterstained with Mayer Hematoxylin (Sigma-Aldrich). After washing, sections were permanently mounted with Aquatex (Merck # 1.08562.0050).

5. Leydig and Sertoli cell number

The number of Sertoli cells Sox9-positive and Leydig cells 3βHSD/CYP17A1-positive was determined counting 20 optical randomly chosen fields of 0.28 mm² area for each IHC experiment, using both light microscope (Zeiss) (40X objective) and scans taken with NanoZoomer 2.0 HT (Hamamatsu Photonics, Herrsching am Ammersee, Germany) followed by analysis using the software NDP view version 1.2.36 (Hamamatsu Photonics).
Both longitudinal and cross sections of tubules in an area of well-preserved testicular parenchyma, were used for scoring. Moreover, the Leydig positive cells were counted only when cut through their geometrical centre (when was visible nucleus or nucleolus).

6. Organ cultures

We used fresh and/or cryopreserved fragments of testicular biopsies to perform organ cultures. Four fragments of 2-3 mm cut with a blade were cultured in immersion in media, composed by α-mem (Lonza, Basel, Switzerland) added with glutamin 0.2M (Sigma-Aldrich), non-essential amino-acids (GIBCO, Thermo Fisher Scientific), penicillin-streptomycin (GIBCO, Thermo Fisher Scientific), gentamicin 10mg/ml (GIBCO, Thermo Fisher Scientific) and Hepes 1M pH 7.7 (Sigma-Aldrich), for 3h, 8h and 24h.
Tissues were cultured at 34 °C in 5% CO_2 with or without 100pM, 100nM hrLH (Luveris 75 UI, Merck Serono, Darmstadt, Germany) and rhCG (Ovitrelle 250 micrograms, Merck Serono, Darmstadt, Germany). After 3/8/24h media were collected for LC/MS-MS analysis and the fragments were dry frozen at -20°C.

7. Protein extraction and quantification

Androgen production was normalized per milligram of proteins that were extracted and quantified by bicinchoninic acid assay (BCA).
Four fragments dry frozen were thawed and the protein extraction was performed by hand homogenization of tissues in home-made Ripa buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH 8 and protease inhibitors Aprotinin 10 mg/ml, leupeptin 10 mg/ml, pepstatin 4 mg/ml, 100mM PMSF). The protein lysates were sonicated (20% amplitude) in ice for 1 minute (10” pulse, 5” pause) and centrifuged for 5 minutes at 13000 rpm. Supernatants were then collected in a new eppendorf. The quantification of proteins was performed with the BCA assay kit (Pierce) following manufacturer's instructions.

8. Androgen measurement by mass spectrometry

Androgens secreted into the culture media, were measured by Dr.ssa Fanelli at the Dept. of Medical & Surgical Sciences, and Centre for Applied Biomedical Sciences, University Alma Mater Studiorum, S. Orsola-Malpighi Hospital, Bologna.
Briefly, the samples were extracted with a mixture of N-hexane: ethyl acetate (8:2) (V/V%) and centrifuged to obtain the stratification of the immiscible phases aqueous and organic. The organic layers were evaporated and reconstituted extracts were placed in HPLC Series 200 system (PerkinElmer, Waltham, Massachusetts, USA). A volume of 75 µL of the reconstituted extract was injected into the system and purified in a column perfusion POROS R1/20 2.1x30mm (Life Technologies, Carlsbad, California, USA) using a 20% methanol flow rate. The separation of the analytes was obtained through a chromatographic gradient composed of mobile phase A (H2O with addition of 0.01% formic acid) and mobile phase B (acetonitrile with addition of 0.01% formic acid). Subsequently, the analytes eluted from the chromatographic system were revealed by positive electrospray ionization type (ESI) by the mass spectrometer API 4000 triple quadrupole-Qtrap (AB-SciexFramingham, Massachusetts, USA) operating in multiple reaction monitoring (MRM). The quantitative

analysis was performed by isotope dilution that requires the use of the analyte identical molecule of interest, the IS (internal standard), by modifying certain atoms in defined positions with their respective stable isotopes: these changes make the different molecular weight of the compound preserving the chemical-physical characteristics. The IS is added in known concentration to all the unknown samples, quality controls and calibrators before any pre-analytical procedure. The quantitative analysis of a chromatographic analysis requires the measurement corresponding to the area under the analyte peak. The calibration curve was generated by the theoretical concentrations of each point. The relation of analyte area/IS area observed in each unknown sample was interpolated on the calibration curve to determine the concentration of corresponding analyte.

9. Enzyme-linked Immunosorbent Assay (ELISA)

The Testosterone and 17OH-Progesterone levels secreted into the culture media were detected also by Testosterone (ADI-900-065, ENZO Life sciences) and 17OH-Progesterone ELISA kits (KA4015, ABNOVA) following the manufacturer's instruction.
Both ELISA kits are characterized by a competitive immunoassay for the quantitative determination of T or 17OHP in biological fluids. Both kits use a monoclonal antibody (T) or a polyclonal antibody (17OHP) against T or 17OHP to bind, in a competitive manner, T or 17OHP in the standard or sample or an alkaline phosphatase molecule which has T or 17OHP covalently attached to it. After simultaneous incubations at room temperature the excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow colour generated is read on a microplate reader at 405nm for T and at 450nm for 17OHP. The intensity of the bound yellow colour is inversely proportional to the concentration of Testosterone in either standards or samples. The measured optical density is used to calculate the concentration of the two androgens.

10. RNA extraction and cDNA synthesis

Total RNA extraction was performed using Tri-Reagent® (Sigma) for each donor sample following the manufacturer's instructions. RNA concentrations were measured at Nanodrop 1000 (Thermo Fisher Scientific) and then 2µg of total RNA were retrotranscribed with High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific) in a final volume of 20µl (10µl buffer, 1µl Reverse transcriptase and 9µl of H_2O for PCR + 2µg of RNA). The reaction was carried out for 60' at 37°C, then 5' at 95°C to stop reaction.

11. qRT-PCR

qRT-PCR assays were performed with SYBR Green (Euroclone®) on a 7500 Real-Time PCR System (Applied Biosystems, Life Technologies®) in 20 µl reactions. Each reaction consisted of 10 µl 2X FluoCycle II™ SYBR® Master Mix, 20 ng of cDNA, final concentration of 300 nM each forward and reverse primers. Transcript levels were measured with two-step thermal cycling conditions: initiation at 95°C for 5', then 45 cycles of 95°C for 15" and 60°C for 60" (fluorescence measurement).
Primers used are listed below:

RPS29 (h) FWD	CGCTCTTGTCGTGTCTGTTCA
RPS29 (h) RV	CCTTCGCGTACTGACGGAAA
GAPDH (h) FWD	TCAACGACCACTTTGTCAAGC
GAPDH (h) RV	GGTGGTCCAGGGGTCTTACTC
3β-HSDII (h) FWD	CCACACCGCCTGTATCATTG
3β-HSD II (h) RV	TCCAGAGGCTCTTCTTCGTG
17β-HSDIII (h)FWD	GATCGAGCGGACTACAGGGA
17β-HSDIII (h) RV	GGCTTGGGAGAAGGTTTGGA
StaR (h) FWD	GGCATCCTTAGCAACCAAGA
StaR (h) RV	TCTCCTTGACATTGGGGTTC
LhR (h) FWD	CTGTGCTTTTAGAAACTTGCCA
LhR (h) RV	TTCACTCTCAGCAAGCATGGA
Cyp11a1 (h) FWD	CCTGTTCCGCTTTGCCTTTG

Cyp11a1 (h) RV	GACGCTGGTGTGGAACATCT
Cyp17 (h) FWD	TGGCCCCATCTATTCGGTTC
Cyp17 (h) RV	AGAGTCAGCGAAGGCGATAC
INSL3 (h) FWD	AGCCAGGAGGCCTGCGAC
INSL3 (h) RV	CAGCCACTGAGGCAGCAGT

Data were analysed with 2^-ΔΔCt method (Livak et all, 2001).
We firstly tested a panel of genes that Svingen T et al (2014) [69] described as the best endogenous normalizing genes for the human testis, in particular ribosomal protein S20 (RPS20), RPS29 and GAPDH gene. We found that both RPS29 and GAPDH expression had an acceptable degree of variability among our donors so we decided to use RPS29 as the housekeeping gene (data not shown).
Then, we arbitrary decided to use donor #20, 50 y/o as the calibrator for the qRT-PCR analysis, because this donor has an intermediate age between young and elderly donors.

12. Statistics

The results of all experiments were expressed as mean ± standard error mean (SEM).
Initial correlations between data sets were analyzed using the Pearson correlation coefficient except when data were visually assessed to differ markedly from a bivariate normal distribution; in that case, Spearman rank correlation was used instead, with P value calculated by a randomization test.
For testing differences between groups T-Student was used except when data were visually assessed to differ markedly from a bivariate normal distribution; in that case, Mann-Withney test was used.
All the statistical analyses were carried out using Sigma Plot 11 software.
P-value < 0.05 was considered significant.

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PUBLICATIONS AND ORAL COMUNICATIONS

Pipolo S, Puglisi R, Mularoni V, Esposito V, Fuso A, Lucarelli M, Fiorenza MT, Mangia F, Boitani C, *Involvement of sperm acetylated histones and the nuclear isoform of Glutathione peroxidase 4 in fertilization*. J Cell Physiol. 2018; 233: 3093-3104. (doi: 10.1002/jcp.26146).

Mularoni V, Esposito V, Boitani C - Regulation of human steroidogenesis during aging – FISV XIV CONGRESS, Rome, 20-23 September 2016 - **SELECTED ORAL PRESENTATION.**

Mularoni V, Esposito V, Boitani C - Leydig cell aging in the normal human testis – 10Th NYRA MEETING, Brussels, 11-14 September 2017 - **SELECTED ORAL PRESENTATION.**

Mularoni V, Esposito V, Boitani C – Changes in Leydig cell number and function during aging in men - 20Th European Testis Workshop, Óbidos, Portugal, 23-27 May 2018 – **SHORT TALK.**