POLYTHECNIC UNIVERSITY OF MARCHE

FACULTY OF MEDICINE

Doctoral School on HUMAN HEALTH

DEPARTMENT OF CLINIC AND MOLECULAR SCIENCE

LABORATORY OF HISTOLOGY AND ENDOCRINOLOGY

"MITOCHONDRIAL AND OXIDANT PROFILE IN MALE INFERTILITY:

TFAM AND GPx-1 GENE EXPRESSION IN HUMAN SPERM"

Coordinator Pr. Mario Guerrieri

Tutor: Pr. Giancarlo Balercia **Dott.ssa** Hana Attia

2021/2022

Abstract

Spermatozoa are particular cells sensitive to oxidative stress (OS) which could be defined as an imbalance between the production of reactive oxygen species (ROS) rate and the antioxidant capacity of seminal plasma. Numerous studies suggest that OS should be considered as a possible idiopathic cause. Energy generation and apoptosis are two of mitochondria's main roles in spermatogenesis. The different physiological elements of reproductive function, from spermatogenesis to fertilization, are significantly regulated by mitochondria. In our study we are interested in two major antioxidant genes; mitochondrial transcription factor A (TFAM) is a very important molecule in initiating mtDNA transcription and Glutathione peroxidase-1 (GPx-1) is an intracellular antioxidant enzyme that enzymatically reduces hydrogen peroxide to water to limit its harmful effects. In this context for the first time, we are interested to detect the TFAM and the GPx-1 gene expression on the spermatozoa by Q-PCR reaction, examining the antioxidant status in asthenozoospermic and asthenoteratozoospermia groups, and exploring the relationship between the 2 genes in the pathology. Our study was carried out on 35 semen samples taken from asthenozoospermic and asthenoteratozoospermia patients, who consulted the laboratory of endocrinology, after sexual abstinence of 4-5 days. These samples were compared to control samples. The result showed a significant increase in the rate of TFAM gene expression in asthenoteratozoospermia, however, a significant decrease in asthenozoospermia and a downregulation in GPx-1 gene expression in both studied groups compared to control but it’s not significant, Thus it seems that the positive correlation found in our study between the two genes suggesting that TFAM and GPx-1 expression both might be linked to loss of motility and increase of abnormal form in sperm involving a new pathway that its very interesting in the future to confirm with a high number of patients and with other divers technique.

Keywords: Oxidative stress, Asthenozoospermia, Asthenoteratozoospermia, TFAM, GPx-1, Q-pcr

Acknowledgements

Firstly, I would like to sincerely thank the members of the jury who accepted to evaluate this work,

I would like to thank my coordinator Pr. Mario Guerrieri and Pr. Fabiola Olivieri.

I would like to especially thank to my tutor Pr. Giancarlo Balercia for supporting me during the period of thesis. You were able to transmit to me a scientific rigor and patience in this initiation to research.

All my gratitude to Pr. Monica Mattioli Belmonte, who welcomed me into her team and in her lab and was able to share her enthusiasm with me as well as for the advice she gave me.

I would like to express my deep and a very special thanks to Dr. Federica Finocchi for her support and her help during my thesis.

I would like to express my deepest thanks to Pr. Monia Orciani and Dr. Caterina Licini.

I would like to express my thanks and respect to Dr. Gianmaria Salvio and Dr. Raffaella Lazzarini.

Finally, I would like to thank all the members of the research laboratory in the faculty of Medicine, DISCLIMO Department of Clinic and Molecular Science, the member of Histology and endocrinology lab, the members of the endocrinology department specially the nurses in the hospital who contributed to the completion of this work and my family.

Hana Attia

Index

Figures list

Tables list

INTRODUCTION

Infertility affects 48.5 million couples globally, and 50% of cases are caused by male factors. (Agarwal et al. 2015). Male infertility is pathophysiologized by a few interconnected endogenous mechanisms. According to reports, oxidative stress (OS) is a key mechanism affecting male reproductive parameters in about 50% of cases of idiopathic infertility in men (Alahmar et al. 2021).

The intricate multi-step process of mammalian spermatogenesis is supported by a small number of spermatogonial stem cells (Hara et al. 2014, de Rooij; 2017). Proliferation, meiosis, and differentiation are the three distinct functional phases that define spermatogenesis. Additionally, asthenozoospermia (low motility) and teratozoospermia (the modification of sperm morphology) are the two major factors that affect male reproduction; therefore, abnormal semen quality is the main cause of male infertility. The presence of oxidative stress (OS) considered one of the primary causes can leads to these alterations (Fainberg & Kashanian; 2019). Reactive oxygen species (ROS) concentrations that are extremely high can surpass natural antioxidant capacity and reach pathological levels. While high ROS levels have a negative impact on male fertility, physiological ROS concentrations are necessary to mediate proper sperm activities (Agarwal et al. 2022).

The cellular machinery, which includes the TCA cycle, oxidative phosphorylation (OXPHOS), and ATP production, is one of the crucial aspects of mitochondrial regulation in postnatal germ cell development during spermatogenesis. These functions must be precisely regulated in a stage-specific manner to support crucial transitions. As a result, spermatozoa use glycolysis for survival but need OXPHOS and glycolysis both for motility and fertilization. Although, during spermatogenesis, there are reductions in the number of mitochondria and changes of their morphology, size, and localization (De Martino et al. 1979; Hess et al. 1993; Meinhardt et al. 1999; Vertika et al. 2020), it is not yet known how altered mitochondrial metabolism influences the fate of germ cells through epigenetic regulation and signaling pathways.

Mitochondrial disorders in germinal tissues have received little attention. Idiopathic asthenozoospermia may be caused by more specific mitochondrial dysfunctions, according to some data. Sperm motility is positively correlated with the activities of

certain mitochondrial enzymes, and this suggests that mitochondria may be essential for maintaining spermatozoa's ability to move (Mackenna; 1995, John et al. 1997, Ruiz-Pesini et al. 1998). The midpiece of an adult mammalian spermatozoon has around 75 mitochondria (Otani et al. 1988). There is compelling evidence that lower human male fertility is related to mitochondrial dysfunction (Folgero et al.1993, Kao et al.1995, Mundy et al.1995, Carra et al. 2004, Sousa et al.2011, Amaral et al. 2013, Hamada et al. 2013, Demain et al.2017, Wu et al. 2019, Vertika et al. 2020). In the middle of the flagella of spermatozoa, "intermediate" type mitochondria are helically stacked end to end. Certain asthenozoospermic subjects have been reported to have fewer mitochondrial gyres and a shorter midpiece length when compared to a reference group (Mundy et al. 1995, Cardullo and Baltz; 1991).

Human mitochondrial DNA (mtDNA) is a circular, double-stranded molecule with a size of roughly 16 kb that contains the genetic information for 22 tRNAs, 2 ribosomal RNAs, and 13 polypeptides (Anderson et al. 1981, Bibb et al. 1981). mtDNA replication happens independently from nuclear DNA (Bogenhagen & Clayton 1977). Nuclear DNA encodes one of the mitochondrial proteins (Reichert & Neupert 2002, Chacinska et al. 2009); for instance, the DNA polymerase gamma POLG is brought in by the nuclear DNA-coded protein known as mitochondrial transcription factor A (TFAM), which then activates mtDNA transcription and causes mtDNA to be packaged into nucleoids (Fisher & Clayton; 1988, Kanki et al. 2004).

TFAM disruption results in embryonic mortality and reduces the number of copies of mtDNA in mice (Larsson et al. 1998), but increased TFAM protein levels in animals with mitochondrial DNA mutations partly restore mitochondrial functioning (Jiang et al. 2017). Oxidative damage and mutations are more likely to affect the mitochondrial genome than the nuclear one. Sequence-specifically and randomly. For mitochondrial transcription to begin, sequence-specific binding to mtDNA promoter regions is necessary, which may also function as an RNA-primer for replication starts. TFAM as well compact the genome by binding mtDNA in a sequence-independent manner (Kang et al.2018), Besides, it has been discovered that MdTFAM transcription aids in Musca domestica version and interim repair of mitochondrial dysfunction and oxidative stress response. Since environmental toxins can up-regulate the transcription of the MdTFAM gene, it is believed that this gene can be employed as a biomarker to detect oxidative stress brought on by these pollutants (Feng et al. 2020).

The primary source of oxidative stress in cells is an explosion of reactive oxygen species (ROS), which is produced mostly in mitochondria (Taverne et al. 2018). As a result, oxidative stress and alterations in mitochondrial function are intimately connected. By controlling mitochondrial function, TFAM, a crucial protein that controls mtDNA replication, can contribute to cell energy metabolism, activate the immune system, and reduce oxidative stress (Chimienti et al. 2018). Furthermore, much like other living cells, sperm defend themselves against this stress. Antioxidant defense systems, specifically antioxidant enzymes, are present in these cells and appear to be essential for male fertility (Colagar et al., 2009). One of the most important ones is glutathione peroxidase (GPX), a protein present in a variety of living organisms and an antioxidant enzyme that oxidizes GSH to GSSG to protect cells from the ROS rise (Morales et al., 2014); Gpx1 has a selenocysteine (Sec) in its active site and is a selenoprotein. According to Kryukov et al. (2003), there are 25 selenoproteins expressed in humans. Gpx1 is involved in redox activities (Hatfield et al. 2014). Selenium has long been thought to be a crucial component of male fertility (Wu et al. 1973), and according to Gpx4 research, declining sperm progressive motility is linked to impaired mitochondrial membrane potential, cellular abnormalities, and oligoasthenozoospermia (Imai et al. 2001). Moreover, there have been conflicting findings regarding the relationship between Gpx1 polymorphisms and the risk of prostate cancer (Kote-Jarai et al. 2002).

To emphasize a potential relationship between the seminal state and the expression of the two genes in male infertility, this thesis evaluates the mRNA expression of the TFAM and GPx1 genes in asthenozoospermic and teratozoospermic patients.

I. THE ANATOMY OF THE MALE REPRODUCTIVE SYSTEM

Unlike the female reproductive system, most of the male reproductive system is located outside of the body. These external structures include the penis, scrotum, and testicles, instead the epididymis, the vas deferens, the ejaculatory ducts, the prostate gland the urethra, the seminal vescicles and the bulbourethral glands are arrenged internally (Fig. 1). The male reproductive system oversees both sexual and urinary functions.

The purpose of the organs of the male reproductive system is to perform the following functions:

- To produce, maintain, and transport sperm (the male reproductive cells) and protective fluid (semen)
- To discharge sperm within the female reproductive tract during sex
- To produce and secrete male sex hormones responsible for maintaining the male reproductive system

The entire male reproductive system is dependent on hormones, especially follicle-stimulating hormone (FSH) necessary for sperm production (spermatogenesis) and luteinizing hormone (LH) which stimulates the production of testosterone, necessary for sperm production too

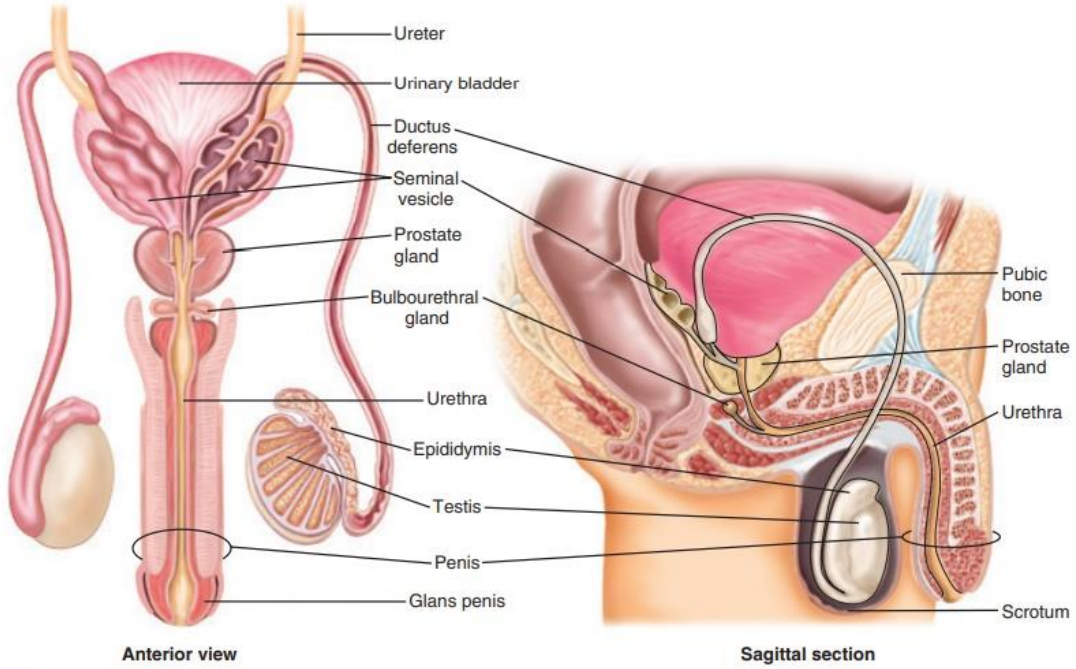


FIGURE The male organs of reproduction.

Fig 1: The anatomy of the male reproductive system

a. Spermatogenesis

Spermatogenesis is the combined process of cell proliferation, meiotic division, and cell maturation. It takes place, according to a precise spatial organization, within the testicular parenchyma (Kerr et al. 2006).

This structure consists of the seminiferous tubules and the stroma, which surrounds the tubules and contains the Leydig cells with endocrine function. The seminiferous tubules are structures organized in a convoluted way, where spermatogenesis takes place, that flows into the rete testis, located at the level of the testicular mediastinum. The efferent ducts branch off from the rete testis and converge to form the epididymis.

The wall of the seminiferous tubules is made up of germinative epithelium, which includes, next to the germ cells in different differentiation states, the Sertoli cells, non-spermatogenic support cells, with important endocrine functions. Germ cells in an early stage of development are found peripherally, while those in the late stages protrude towards the lumen.

The process by which the germinative cellular elements pass from the periphery to the lumen lasts about 74 days (Fig 2) and includes three phases (Kierszenbaum et al. 1994)

- a phase of mitotic proliferation, which involves the spermatogonia.
- a meiotic phase, carried out by the spermatocytes, which allows the division of the genome into haploid cells (spermatids) and during which all the processes related to genetic recombination (crossing-over) also take place.
- a final phase of cell differentiation without further division, which leads to the formation of the mature sperm, through a series of morphological changes known by the term spermiogenesis.

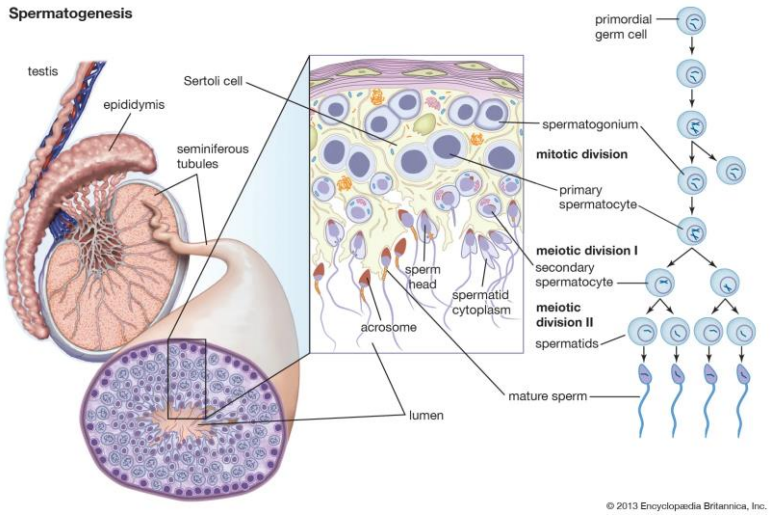


Fig. 2 Schematic human spermatogenesis

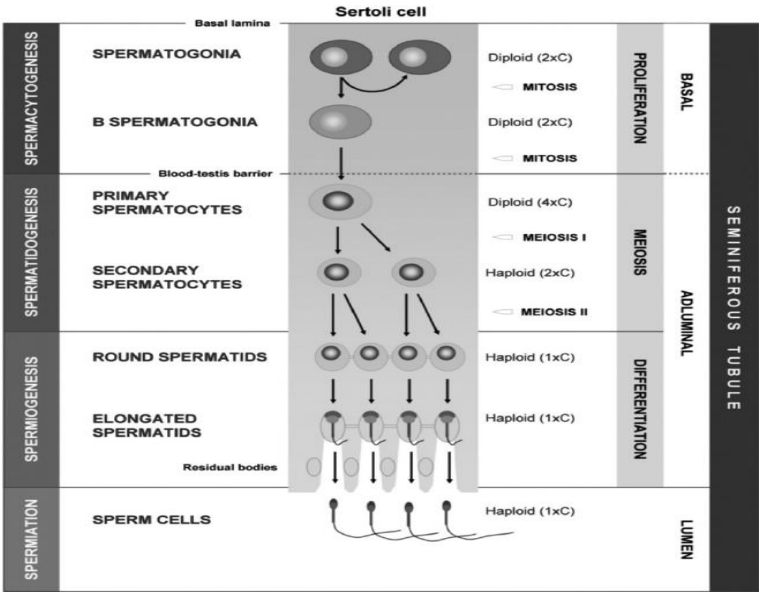


Fig 2: Different phases of the spermatogenesis

b. Mitotic phase

Spermatogonial stem cells represent the mitotic phase of spermatogenesis that precedes meiosis and, therefore, the obtaining of cells from the haploid chromosomal set. This phase lasts 8-10 days in various mammals and has the aim of increasing the pool of cells destined to become spermatozoa. There are three types of spermatogonia: Ad (dark) stem spermatogonia divide to form other Ad stem spermatogonia or to give rise to proliferating spermatogonia, Ap (pale) spermatogonia, from which B spermatogonia, cells destined to differentiate, originate. By observing histological sections of seminiferous tubules under the microscope, it is possible to note that stem spermatogonium has two different mitotic behaviors. In fact, when it divides to give rise to other stem cells, after the telophase, the cytodieresis is complete and leads to the formation of two single daughter cells, that is, completely separated from each other. If, on the other hand, the stem spermatogonium must give rise to cells destined for the proliferative and differentiative pool, the cytodieresis is not completed. This disruption in the normal cell division process results in the two daughter cells being joined by a cytoplasmic bridge. This pair of cells divides even more times, always without completing the cytodieresis, giving rise to chains of cells always joined by cytoplasmic bridges. The cellular syncytes thus obtained will remain such for the entire duration of spermatogenesis. Only now spermiation, or rather when the spermatozoa are released

into the lumen of the seminiferous tubule, the mature cells will separate from each other, acquiring their own individuality.

c. *Meiosis phase*

As mentioned, only type B spermatogonia undergo the process of meiosis that will lead to the generation of male gametes. From type B spermatogonia, therefore, primary, or first-order spermatocytes originate, which duplicate their DNA and subsequently go encounter the first meiotic division which, in mammals, lasts from 13 to 18 days and ends with the formation of secondary or second-order spermatocytes. These cells, present for only 8 hours, are endowed with a haploid but doubled chromosomal set due to the persistence at this stage of two chromatids for each chromosome of the genetic set. It is with the second meiotic division that the two sister chromatids of each chromosome segregate each in the nucleus of one of the two daughter cells, now called spermatids.

d. Spermiogenesis

Spermiogenesis is a process lasting 13-16 days through which the spermatids, without further divisions, become spermatozoa developing specific structures such as the flagellum and the acrosome, essential for the ability to fertilize. Initially, the spermatid has a small spherical nucleus, 5-6 mm in diameter, with finely scattered chromatin. The Golgi apparatus is adjacent to the nucleus and the two centrioles, mitochondria, and other organelles are in the periphery of the cell. Spermiogenesis can be divided into four phases: the Golgi phase, the cap phase, the acrosomal phase, and the maturation phase. During these stages of development, there are deep changes, both morphological and functional.

During the Golgi phase, small granules contained in a vacuole appear in this apparatus, called proacrosomal granules. Thereafter, the proacrosomal granules merge to form a single acrosomal bead wrapped in a membrane that is part of the Golgi complex. In the cap phase, the acrosomal vesicle increases in volume and prepares to cover the anterior two-thirds of the nucleus, thus forming the acrosome cap. In the meantime, the two cellular centrioles move towards the pole of the nucleus opposite the newly formed acrosomal cap: the so-called proximal centriole, takes position at the posterior pole of the nucleus; the other, the distal centriole, acts as a basal corpuscle for the flagellum which, in the meantime, is being organized. During the subsequent stages of

differentiation, the distal centriole disappears. In the subsequent acrosomal phase, considerable changes occur in the acrosome, nucleus, and flagellum. The acrosomal apparatus adapts to the nuclear form and covers a large part of this, placing itself in the opposite direction to the flagellum. The nucleus, in turn, moves towards the periphery of the cell, taking a more elongated shape. The chromatin inside condenses and becomes inactive. The cytoplasm stretches at the level of the caudal pole of the nucleus to envelop the proximal part of the flagellum, where the mitochondria are concentrated, which will then surround the axoneme in that portion, creating a real collar. During the maturation phase, the spermatid completes its transformation into sperm in all its aspects: the nucleus and the acrosome assume the characteristic shape of the species; in humans, the nucleus is pear-shaped, and the acrosome covers the anterior two-thirds.
In the final stages of spermiogenesis, there is a deep reorganization of the chromatin which condenses further becoming extremely compact. The procedure through which this occurs has the purpose of condensing the chromosomes in the very narrow nuclear space of the spermatozoon and protecting this chromosomal kit from the insults that threaten it during its transit along the male and female genital tracts.

e. Spermiation

At the end of spermiogenesis, the cytoplasmic bridge that holds the cell which has reached maturity together with the cell chain deriving from the same clone is eliminated, forming the so-called residual bodies immediately phagocytosed by the Sertoli cells. At this point the spermatozoon, which has become a single cell, is released into the tubular lumen, from where it is rapidly pushed towards the rectus tubules and the rete testis thanks to the peritubular myoid cells and their contractile activity. A small portion of cytoplasm remains attached to the sperm, the cytoplasmic drop, which will be eliminated during the maturation of the sperm in the epididymis.

II. Male infertility

1. Definition

According to World Health Organization, infertility is a disease of the male or female reproductive system defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse (WHO 2010).

Infertility affects millions of people of reproductive age worldwide and has an impact on their families and communities. One out of 6 couples consults for procreation difficulties, the prevalence is estimated at approximately 15 % of couples, attempting pregnancy and in half of these cases, infertility can be attributed to being due to male factor. Male infertility is mostly idiopathic, and its frequency is estimated at 40-75% (Dohle et al., 2002).

2. *Semen analysis*

- ✓ Teratozoospermia

The morphology of the spermatozoon constitutes an important parameter in the exploration of the quality of the sperm for infertile men, indeed, the spermatozoa is a cell obtained following a long process of differentiation and maturation. Each of these stages could be altered, resulting in the production of abnormal sperm; this is called teratozoospermia, in this case, the pathological threshold depends on the classifications used (De Braekeleer et al. 2015).

- ✓ Asthenozoospermia

Asthenozoospermia is characterized by a drop in the mobility of spermatozoa (SPZ). Mobility is considered normal beyond 40 %. It's considered primary if the drop in mobility occurs within the first hours or considered secondary if it is 4 hours after sperm emission. On disting total asthenozoospermia; the total absence of motility of all the SPZ (Afzelius et al.1975).

As the etiology and pathogenesis of asthenospermia are complicated, there still are some arguments. In the past several decades, most of the studies of asthenospermia's treatment are focused on the regulation of hormones, antioxidation, (Busetto et al. 2018) providing energy for sperm, (Liu et al. 2020) supplying trace elements, (Alsalman et al. 2018) and assisted reproductive technology (ART). Exploration of male infertility

There are 3 groups dividing the Asthenozoopsermic pathology.

1. Moderate Astheno. 30 % <a+b<40 %
2. Severe Astheno.10 % <a+b<30 %

3. Extreme Astheno. a+b<10 %

a: the percentage of rapid progressing motile spz

b: the percentage of slow-progressing motile spz

In the case of important Asthenozoospermia, several causes can be evoked like infection, hyperviscosity, flagellar dyskinesia, auto-immunization…

- ❖ Exploration of male infertility

Male infertility is defined by the presence of abnormalities in sperm parameters, according to the criteria defined by the WHO (who.2010).

Variables	Normal parameter
Semen volume (ml)	1.5
Total sperm count	39 (million)
Sperm concentration	15 (million/mL)
Total motility	40 (%)
Progressive motility	32 (%)
Vitality	58 (vital sperm, %)
Sperm morphology	4 (normal forms, %)
pH	>7.2
Peroxidase-pozitive leucocyte	<1.0 (million/ml)

Tab1: Official norm of sperm parameters who 2010

3. Principal etiology

The etiologies of Male infertility are principal:

- Endocrinal pathology
- Nonobstructive pathology (secretory)
- Obstructive pathology
- Ejaculation pathology
- Varicocele
- Idiopathic oligoasthenoteratozoospermia(OAT)
- The presence of anti-sperm antibodies.

4. Varicocele

Varicocele is defined as a dilation of the pampiniform plexus in the testicle, it's found in 15 % of men with normal sperm parameters, and in 25-40% of men with sterility (Sharlip et al. 2002). Its multifactorial pathology, several mechanisms have been proposed to explain infertility, but none is proven. Suggested mechanisms include hypoxia and stasis, testicular venous hypertension, increased temperature and increase catecholamines in the spermatic vein and increase oxidative stress (Marmar; 2001).

5. Spermogram

Spermogram is currently basic examinations to assess the characteristics of the sperm. They are straight the other complementary examination which will identify one or several causes of infertility.

- Spermogram: it represents the basic examination that allows a qualitative and quantitative analysis of the sperm, obtained after masturbation in the laboratory to identify a biological cause of infertility.

- Spermocytogram: it's the usual name to define the morphological analysis of human spermatozoa which makes it possible to evaluate the percentage of atypical forms (AF).

The classification used in the laboratories.

	DEFINITION
NORMOZOOSPERMIA	The parameters in the sperm are within the reference values of the WHO standards
OLIGOZOOSPERMIA	The sperm parameters with the concentration of spermatozoa are lower than the WHO reference value
ASTHENOZOOSPERMIA	The parameters of motility are lower than the WHO reference value
AKINETOZOOSPERMIA	Absence of motility
TERATOZOOSPERMIA	The number of abnormally shaped spermatozoa is greater than the WHO reference value
AZOOSPERMIA	Absence of spermatozoa in the ejaculation
ASPERMIA	Absence of ejaculation
CRYPTOZOOSPERMIA	A very low number of spermatozoa were obtained in the ejaculate.

Tab 2: Definition of terms relating to sperm parameters.

III. Oxidative stress

One of the main causes of infertility in men is the high production of reactive oxygen species (ROS). Like any other cells, sperm cells are highly susceptible to oxidative injury, it can also be provoked by the reduced antioxidant capacity of semen and spermatozoa creating the conditions termed of oxidative stress (Browne et al. 2008).

1. Definition

Oxidative stress is defined as an imbalance between pro-oxidant or ROS and antioxidants (Rahal et al. 2014). Recently, this notion was modified by defining itself as an imbalance between the biochemical processes of ROS production and those responsible for their control (Powers et al. 2010).

Haploid sperm cells are poorly equipped to fight free radical-mediated attacks. In addition, the high polyunsaturated fatty acids (PUFA) content of the spermatozoa membrane makes them more susceptible to ROS attacks. Intriguingly, Sperm cells are amongst the first cells that were shown to be good producers of ROS (Rashki Ghaleno et al. 2021) and spermatozoa-produced ROS have been found to be the key players in signal transduction pathways leading to sperm capacitation (Aitken et al. 2014).

To prevent oxidative stress, there are enzymatic and non-enzymatic antioxidant defense systems in semen such as superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase, and vitamins (Huang et al. 2018).

2. Reactive oxygen species (ROS)

The oxygenated free radicals are molecules characterized by the presence of at least one unpaired electron and a very short lifespan; they are in fact unstable molecules and react with other molecules by stripping them of an electron or by transforming them into radical molecules. They can lead to tissue damage by capturing electrons from a stable molecule. We distinguish a very wide set of radicals namely, superoxide anion (o2--), hydroxyl radical (OH) but also certain non-radical oxygenated derivatives whose toxicity is significant, such as hydrogen peroxide (H2O2) (Miranda-vilela et al. 2010).

Several methods are used to directly estimate the rate of ROS in the sperms or indirectly by measuring the oxidation, they are dosed in chemiluminescence, using a luminometer,

and they can be also detected by staining with Nitro blue tetrazolium (NRT) which produces a blue formazan, observable in photonic microscope following the reduction of superoxide ions by the tetrazolium.

ROS production

They are molecules derived from the aerobic metabolism of cells, detected for the first time in semen by Macleod in 1743. Different cellular elements can generate, the most widely accepted is the mitochondria via the complex of the respiratory chain. Leucocytes under the action of inflammatory stimulus can discharge up to 100 times more than usual ROS (Saleh et al. 2003). Apart from leucocytes, spermatozoa themselves are a potential source of ROS, especially when they exhibit morphological alterations. During spermatogenesis, elongated spermatids lose their cytoplasm, and they acquire the necessary condition for fertilizing power. However, damaged spermatozoa may retain excess cytoplasm around the intermediate part. We speak of excess resident cytoplasm (ECR), this system activates NADPH, resulting in ROS production (Rengan et al. 2012).

ROS can also be produced access during exposition of the body to various exogenous and endogenous factors or stress such as radiation, alcool, tabac, and high temperature (Methorst et al. 2014).

Physiological role of ROS

Species derived from the active oxygen (superoxide anion and hydrogen peroxide) are physiological compounds involved in many normal processes. They are an essential factor in the capacitation of spermatozoa. Hydrogen peroxide H2O2 seems to control the Tyrosine phosphorylation associated with spermatozoa capacitation (Rivlin et al. 2004).

The deleterious effect of ROS

If ROS reacts with a cellular compound before being removed by antioxidant molecules, it can cause irreversible damage to the cells. In case of oxidative stress, the high concentration of ROS can lead to the death of a cell by apoptosis (ROS can activate the mitochondrial signaling pathway for apoptosis), the preferred targets of ROS are lipids, proteins, and principally nucleic acids (Aitken et al. 2014). Indeed, ROS affects the DNA quality; inducing the DNA fragmentation (Aitken et al. 2014).

3. The DNA of spermatozoa

Reproduction in males is characterized by the production of large numbers of sperm through the process of spermatogenesis wherein the phenotypic normality gamete and genomic integrity may be altered (Aitken and Krausz; 2001). The formation of mature sperm is a unique process involving a series of meiosis and mitosis, cytoplasmic change in the architecture, replacing somatic histones with transition proteins, and final addition of protamine, leading to a highly compacted chromatin.

a) Spermatic DNA structure

The compaction refers to the complex and specific structure in which the DNA of sperm ejaculated is highly condensed. Sperm chromatin is a highly organized structure, the compacted structure is composed of DNA and a heterogeneous nucleoprotein (Evenson et al. 2002). In somatic cells, DNA is organized into nucleosomes, this nucleosome consists of a histone octamer around which the DNA molecule is wound. Two models are currently available to describe the compacted structure of DNA, a zigzag pattern, or a solenoid-shaped model (Dorigo et al. 2004). The chromatin of the spermatozoon is organized in a superposition of DNA loops. These are shaped like a ‘doughnut’

b) Chromatic rearrangement of germ cells

It was during spermiogenesis that histones type nucleoproteins are replaced by a structure based on protamines to reduce the nuclear volume and increase the compaction of DNA.

Chromatin spermatozoa are about six times more condensed than somatic cells. This very important compaction corresponds to the implementation of physical protection of the core gamete against the various attacks to which it could be submitted along its path, the seminiferous tubules to the oocyte cytoplasm. Finally, in mature human spermatozoa, about 15% of histone persists in the nucleus and over 70% of basic nuclear proteins are protamine (Braum; 2001; Sergerie et al. 2005).

4. Tests of appreciation of the quality of chromatin of human spermatozoa

Most techniques to seek DNA fragmentation will hardly distinguish between that due to a lack of maturity or final compaction of chromatin, and that due to endogenous and exogenous causes of DNA fragmentation. We distinguish between cytochemical types of tests for the characterization of chromatin (with fluorochromes) and biochemical tests (Roux et al. 2004).

- Toluidine blue staining (BT)

The level of condensation of the sperm chromatin is evaluated using a dye; toluidine blue or hydrochloride tri-methylthionine. This is a basic dye that has high-affinity basophil molecules including nucleic acids. A highly condensed DNA will leave penetrate little dye, giving a very pale blue core.

- Staining of acetified aniline blue (BA)

The aniline blue stained chromatin of round spermatids. An anomaly of the establishment of definitive nucleoproteins sperm will result in persistence of nuclear staining. The sperm usually considered normal and condensed on the nuclear level will have a completely colorless core (Dadoune et al. 1988).

- Analysis of sperm chromatin structure (SCSA)

This technique is based on the fact that the abnormal sperm chromatin is more likely to denature after treatment in situ with an acid and/or heat solution. SCSA technique uses meta-chromatic proprieties of orange acridine. The analysis is done with a flow cytometer to measure the ratio of red fluorescence (single denatured DNA) and green fluorescence (double-stranded DNA).

Flow cytometry is used to study many sperm (sperm with a low count cannot be analyzed). The results obtained using the SCSA are only weakly correlated with WHO parameters like concentration, morphology, and motility (Evenson et al. 2002)

- Aridine orange test (AOT)

Acridine orange is an intercalating agent that fluorescence metachromatic green (515-530nm) when it's associated with double stranded DNA and orange red(>630nm) when it's joint single-stranded DNA or denatured (Roux et al. 2004). Coloring with orange acridine sets oeuvre the DNA denaturing property in acidic condition and this technique does not require a flow cytometer.

- Evaluation of chromatin by chromomycin A3(CMA3)

A correlation was found between abnormalities in chromatin condensation and accessibility of the DNA to Chromocycin A3. CMA3 binds to DNA segments rich in guanines and cytokines, areas in competition with protamine binding sites. It reveals a local deficiency of definitive nucleoproteins in the chromatin, and DNA denatured or fragmented areas (Hammadeh et al. 2001).

- Sperm chromatin dispersion (SCD)

This test could be defined as a simple and inexpensive method, it consists in treating spermatozoa with an acid solution and then with lysis buffer. The DNA is then counterstained with DAPI (4'-6-diamidino-2-phenylindol). After this treatment, gametes without fragmented DNA release DNA loops which form large halos (Fernandez et al. 2003). Fertile men have significantly more gametes with large halos than infertile men (Chohan et al. 2006).

5. The ROS regulation system protecting spermatozoa

In order to protect spermatozoa against cellular and nuclear damage caused by the increased production of ROS, an antioxidant regulatory system would be essential to eliminate these molecules. They are two principal systems: the enzymatic and the non-enzymatic system

a) Enzymatic system

This system is the first defense line in our bodies against ROS and they are crucial to preventing membrane lipid peroxidation (LPO), principally we can cite; superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx).

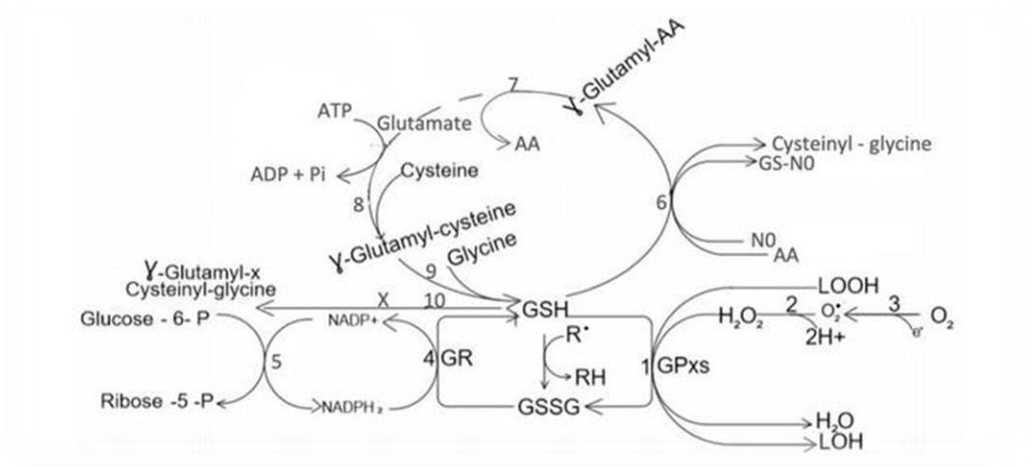
b) Non-enzymatic oxidant system

They are trace elements (zinc, selenium, vitamins…) that play a very important role in male fertility because they constitute cofactors for antioxidant enzymes, and they are involved in the structural integrity of spermatozoa (Colagar et al. 2009).

IV. Glutathione peroxidase (Gpx)

1. Glutathione system

Glutathione, also known as -glutamyl-cysteinyl-glycine (GSH), is an intracellular tripeptide found in all mammalian organs. This thiol-containing compound is found in high amounts in the cytosol (1–11 mM), nuclei (3–15 mM), and mitochondria (5–11 mM) in cell compartments.



.

Fig 4: Glutathione system; the enzymes that catalyze the reactions are (1) GPxs, (2) superoxide dismutase, (3) NADPH oxidase and mitochondrial respiratory complexes, (4) glutathione reductase, (5) gluco-6-phosphate dehydrogenase, (6) γ-glutamyl transpeptidase, (7) γ-glutamyl cyclo-transferase, (8) γ-glutamylcysteine synthetase, (9) glutathione synthetase, (10) γ-glutamyl transpeptidase. Abbreviations: AA, amino acid; O2−, radical superoxide; H2O2: hydrogen peroxide; GS-NO, glutathione-nitric oxide adduct; LOH, alcohol lipid; LOOH, hydroperoxide lipid, R., radical; RH, nonradical; X, electrophilic xenobiotics

Glutathione peroxidase is a selenoprotein with biological functions that include the detoxification of endogenous and exogenous ROS, as well as the elimination of xenobiotics from cells. Mills (1957) was the first who identify the GPX, which was later detected in all cells investigated. Because it was shown to be expressed at large quantities in red cells, it was dubbed cytoplasmic GPX or erythroid-specific GPX.

2. GPx-1 gene

GPX is one of the antioxidant enzymes involved in the recycling of ROS. In the male genital tract, some GPX genes are expressed differently. the GPX protein family is split

into five main classes based on its basic sequence, substrate selectivity, and subcellular location. The dysfunction of this molecule's expression in spermatozoa has been linked to human infertility in several studies (Imai et al. 2001; Foresta et al. 2002). In other studies, GSH is used to catalyze the reduction of hydrogen peroxide and lipid peroxides by eight distinct isoforms of GPx (Brigelius-flohé and Maiorino; 2013, Deponte; 2013). GPx1 contain a selenocysteine residue (SeCys) and it is tetramers (Nguyen et al.2011, Pastori et al. 2016). Furthermore, Gene sequence comparisons and dendrogram analyses have revealed that the multimember family of GPX sequences is most likely derived from an ancestral gene through duplication events and intron-exon shuffling (Ursini et al. 1995).

GPx1 protects cells from cytotoxic peroxide-induced oxidative damage, lipid peroxidation, and protein degradation. Only soluble hydroperoxides like H2O2 and hydroperoxy fatty acids are reduced by GSH cytosolic and mitochondrial peroxidases. Hydroperoxide-induced apoptosis is inhibited by increased GPx1 activity. Most tissues contain GPx1 (Morales et al. 2012, Brigelius-flohé and Maiorino; 2013)

In addition to the ubiquitous expression of the cytosolic GPX1 protein, it appears that the tissues of the male genital tract have evolved specific GPX coverage. Another intriguing feature is that we have only observed peculiar patterns of expression of the different GPX genes in the tissues of the male genital tract (Ekoue et al. 2017).

3. GPx-1 regulation

The redox status of thiol mammalian systems is substantially influenced by GSH (Lu; 2013). Through the GPX activity, which oxidizes GSH to GSSG, and the action of NADPH-dependent glutathione reductase, which produces GSH, GSH acts as an antioxidant, removing free radicals and other ROS. Glutathione-S-transferase activity detoxifies xenobiotics and different metabolites, resulting in mercapturates and reactivated glucose-6-phosphate dehydrogenase. Besides, GPx isoforms catalyze the reduction of inorganic and organic hydroperoxides, to put it simply. During catalysis, glutathione functions as a reducing equivalent, inactivating free (Farhat et al.2018).

Many GPX proteins are found in spermatozoa. Due to its isoform activities and patho-physiological functions, GPx has the status of a redox system (GSH/GSSG) in the

glutathione (GSH) system to prevent oxidative damage. As a result, the GPx is the first line of defense against free radicals in the body. In addition to the oxidation of proteins and deoxyribonucleic acid, its inadequacy generates oxidative stress, which causes inflammation, and metabolic abnormalities (DNA). The most effective biological antioxidant reducer is GPx.

GPx1, a selenocysteine-dependent enzyme, has been the focus of many investigations on the metabolic control of GPx. Exogenous Se regulates human GPx1 enzymatic activity without changing GPx1 mRNA levels, suggesting that Se regulates the human GPx1 gene posttranscriptionally (Chada; 2015). Etoposide, a topoisomerase II inhibitor, apoptosis inducer, and p53 activator all stimulate GPx1 by positively regulating a promoter region upstream of the gene. The p53 signaling pathway to the antioxidant pathway involves the transactivation of GPx1 by p53 links. Furthermore, an investigation of p53-induced apoptosis in a human colon cancer cell line revealed that increased p53 expression was linked to increased GPx1 expression (Puzio-Kuter, 2011)
Direct tissue damage and activation of age-related NF-B inflammatory pathways ensue from GPx deficiency (Erden-Inal and Sunal; 2002, Brigelius-flohé and Maiorino; 2013).

4. GPx-1and male infertility

GPXs are a group of enzymes that differ slightly in their characteristics. The traditional intracellular GPX-1 is widely expressed, and it has a direct association with sperm motility (Dandekar et al. 2002). Besides, GPXs have been of special interest in semen since classical experiments by Alvarez and colleagues demonstrated an increase in membrane damage after the addition of its specific inhibitor mercaptosuccinate (Alvarez et al. 1987). However, knockout mice lacking GPx1 have been found to be fertile (de Haan et al. 1998, Lavoie et al. 2011).
Furthermore, since inhibiting GPX with mercaptosuccinate increases membrane damage, the GPx/GR system has been demonstrated to play a key role in maintaining membrane integrity in human sperm (Alvarez and Storey; 1989).

V. Human Mitochondriale DNA

1. Organization

The human mitochondrial DNA (mtDNA) is a closed circular extranuclear genome of 16,569 pb (Anderson et al. 1981). It is made up of two strands, one guanine-rich heavy (H) and the other cytosine-rich light (L). 13 genes encode important components of oxidative phosphorylation (OXPHOS), 22 genes encode tRNA genes, one for 12s and one for 16s tRNA genes required for mitochondrial protein synthesis, and 37 genes encode tRNA genes. The number of mitochondria and their genome content normally decreases during spermiogenesis to prevent the transmission of the paternal mitochondrial genome to the offspring. This genome is more susceptible to oxidative damage, and mutations are more prevalent than in the nuclear genome, further, the mitochondria are critical organelles not only for energy metabolism, but also for cell apoptosis regulation via the release of cytochrome C and other pro-apoptotic proteins (Thangaraj et al.2003).

In contrast to nuclear genes, mtDNA lacks introns, and the entire coding sequence is continuous. The displacement loop (D-loop), a length of 1121bp that contains the H-strand replication origin and promoters for L and H strand transcription, is the only non-coding portion of mtDNA. Nuclear genes code for the remaining mitochondrial OXPHOS proteins, metabolic enzymes, DNA and RNA polymerases, ribosomal proteins, and mtDNA regulatory factors. The OXPHOS unit is a vital component of mitochondria, which is the primary generator of TP as well as ROS (John and al. 2000, Wallace et al. 2013)

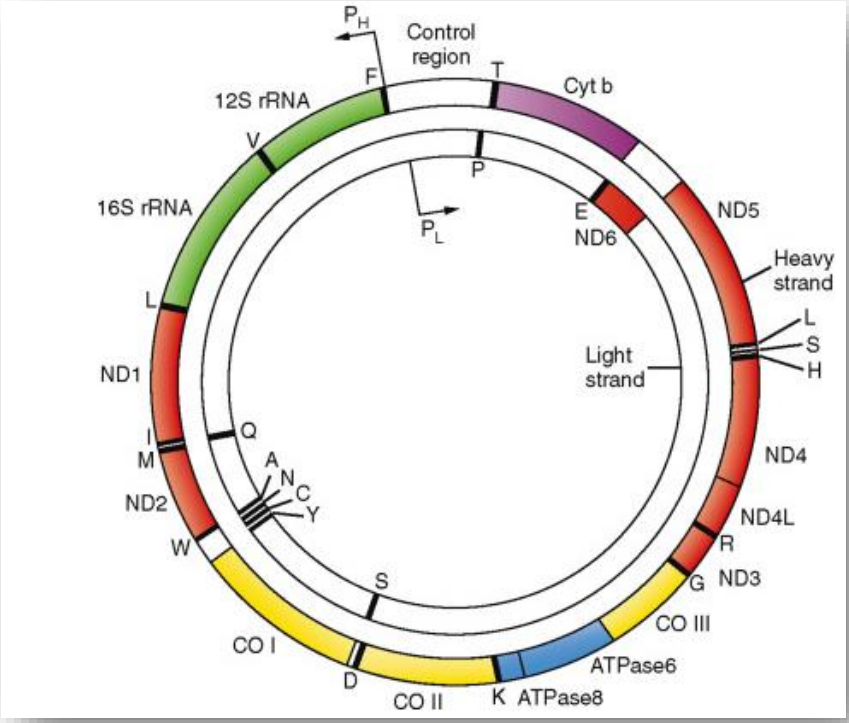


Fig 5: Organization of the human mitochondrial genome.

Human mitochondrial DNA (mtDNA) is a circular double-stranded molecule and contains approximately 16,569 base pairs. The D-loop regulatory region is expanded and shown above. Protein coding and rRNA genes are interspersed with 22 tRNA genes (denoted by the single-letter amino acid code).The D-loop regulatory region contains the L- and H-strand promoters (PL and PH, respectively) along with the origin of H-strand replication (OH).mtDNA transcription complexes containing mitochondrial RNA polymerase, Tfam, and TFB are depicted in the expanded D-loop along with the conserved sequence blocks (CSB I, II, and III).The origin of L-strand replication (OL) is displaced by approximately two-thirds of the genome within a cluster of five tRNA genes. Protein-coding genes include cytochrome oxidase (COX) subunits 1, 2, and 3; NADH dehydrogenase (ND) subunits 1, 2, 3, 4, 4L, 5, and 6; ATP synthase (ATPS) subunits 6 and 8; and cytochrome b (Cytb). ND6 and the eight tRNA genes encoded on the L-strand are in bold type and underlined; all other genes are encoded on the H-strand (Amorim et al. 2019).

Human mtDNA is compact (intronless) and devoid of histones or DNA-binding proteins (Spelbrink et al. 2000). It replicates rapidly without efficient proof-reading and DNA repair mechanisms (Yakes et al. 1997) This lack of an efficient repair system in

mitochondria accelerates the rate of mitochondrial DNA mutation that thought to be 10–100 times higher than in nuclear DNA.

In fact, mitochondria are involved in a variety of processes; ATP generation, cell cycle arrest via the "intrinsic" death route, the creation of reactive oxygen species (ROS), intracellular calcium homeostasis, and the manufacturing of steroidal hormones are all examples. The genome, or mitochondrial DNA, of the mitochondrion is unique (mtDNA) (Spelbrink et al. 2000). Individual sperm maturation by meiotic division of spermatocytes occurs continually during mammalian spermatogenesis. However, whether mitochondrial respiratory activity is required for the meiotic process remains unknown (De Martino et al.1979).

A mammalian spermatozoon includes between 22 and 75 mitochondria in the intermediate region of the flagellum, with the mitochondrial sheath creating a typical helical pattern. Besides, mitochondria perform an important role in the sperm cell, guaranteeing proper flagellar activity and sperm motility, both of which are required for fertilization. Male infertility can be caused by reduced sperm motility (Kao et al.1995, 2004).

Several mitochondrial proteins encoded by nuclear DNA regulate the number of copies of mtDNA, including polymerase gamma (POLG), the replicative helicase Twinkle, and mitochondrial transcription factor A. (TFAM).so mtDNA does not interact with histones, but instead with transcription factor A of the mitochondria (TFAM), which influences transcription and replication (Scarpulla, 2008)

From the literature the transcription, replication, and structural organization of mtDNA are all regulated by TFAM. However, few researchers have looked at the relationship between the expression of TFAM, which is required for the synthesis of the primers required for mitochondrial genome replication, and the number of copies of sperm mtDNA(Campbell et al.2012).

2. Mitochondrial transcription factor A

TFAM is the mitochondrial transcription (previously is mtTF-1 and mtTFA) machinery's third most important component, TFAM attaches to mitochondrial promoters in a sequence-specific manner, forming a stable U-turn in DNA (Shi et al. 2012, Ngo et al. 2011, Jimenez-Menendez et al. 2010). TFAM can bind, unwind, and

bend DNA without sequence specificity, like other members of the high mobility group (HMG)-box domain family. In fact, TFAM coats the entire mtDNA molecule, generating a tight nucleoid structure (Falkenberg et al.2007, Farge et al. 2014, Kaufman et al. 2007).

Biophysical research has also revealed critical new details on how the protein alters the structure of mtDNA. TFAM can slide over DNA and generate longer patches when it collides with other TFAM monomers, according to some research (Farge et al.2012).

The TFAM protein is required to initiate and regulate mitochondrial DNA amplification and biogenesis, as well as to drive mitochondrial DNA transcription (Spelbrink et al. 2000, Yakubovskaya et al.2006).

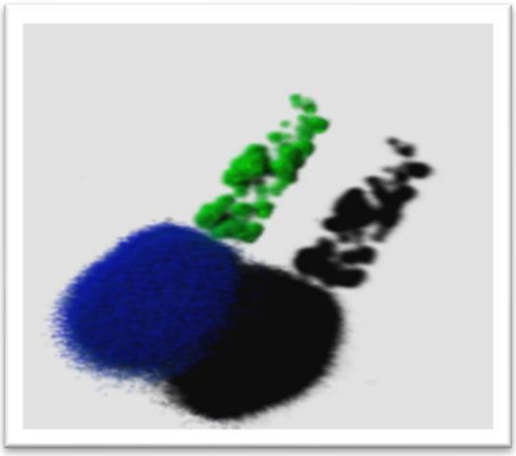


Fig 6: The human sperm midpiece.

Three-dimensional rendering of confocal microscopy images acquired with human sperm stained with an antibody against the mitochondrial protein TFAM, clearly showing the localization and organization of the sperm midpiece (green). DAPI was used as a DNA counterstain for the sperm nucleus (blue). (Amaral et al. 2013)

3. TFAM regulation

Consistent with this finding, human Lon (as a DNA Binding Protein) has been shown to be a protein component of mitochondrial nucleoids, which are large multiprotein complexes bound to mtDNA that support genomic stability, inheritance, and expression. Although the mtDNA binding by Lon in cultured cells has been established, the function of Lon at the mitochondrial genome remains unclear. Results show that Lon degrades transcription factor A of mitochondrial (mt-TFA, TFAM), thus Lon may be

responsible for monitoring the quality control of proteins involved in mtDNA metabolism. However, it is also possible that Lon plays a role in regulating the function of mtDNA maintenance proteins (Matsushima et al. 2010, Jae Lee et al 2013)
Besides, mitochondria participate in diverse processes ranging from spermatogenesis to fertilization to regulate male fertility (Yoo-Jin Park and Myung-Geol Pang; 2021). The mitochondrial RNA polymerase and transcription factor TFAM are responsible for maintenance of the mtDNA copy number in mammals and are regulated by the pathways involving PGC-1α, the mitochondrial transcription specificity factors TFB1M and TFB2M, and the nuclear respiratory factors NRF-1 and NRF-2 (Laura et al.2009, Zhai et al.2019).

4. TFAM and oxidative stress in male infertility

Mitochondria are vital components of spermatozoa that provide energy for movement. Indeed, mitochondria are found in the spermatozoa's mid-piece and provide ATP, which is mostly employed for flagellar motion. Several genes encoding proteins involved in oxidative phosphorylation and ATP synthesis can be found in mitochondrial DNA (mtDNA) (Bafaluy et al. 2003). Histones and DNA-binding proteins are absent from human mtDNA, which is compact (intronless). It reproduces quickly because it lacks effective proofreading and DNA repair mechanisms. In addition, excess of ROS in sperm causes oxidative stress, which impairs DNA demethylation in the paternal pronucleus and inhibits embryo development (Shi et al. 2022)

In infertile men, ROS and mitochondria have been linked to apoptosis, as excessive amounts of ROS damage the inner and outer mitochondrial membranes and can cause the release of cytochrome C from the mitochondria, Cytochrome C protein stimulates mitochondria. Caspases are activated, and apoptosis is induced oxidative stress levels have been found to be substantially greater (Wang et al 2014). In addition, mutations, deletions, and changes in the copy number are all changes that influence mtDNA. Infertility is caused by mutations and deletions in the mitochondrial genome, which decrease sperm motility and/or concentration (Folgero et al. 1993)

In 2007, Amaral show that the low-quality sperm have the lower levels of TFAM (Amaral et al. 2007). The high sperm mitochondrial DNA copy number (mtDNA-CN) could be due to aberrant expression of genes that govern mtDNA transcription and replication, such as mitochondrial transcription factor A (TFAM). Because TFAM

expression is inversely correlated with sperm motility and favorably correlated with mtDNA-CN, Faja et al.2019 show the dysregulation of TFAM expression is accompanied by a qualitative impairment of spermatogenesis (Faja et al.2019).

However, just a few researchers have investigated the sperm mtDNA copy number. Some researchers showed it to be higher in samples with impaired motility, concentration, or sperm morphology (Bonanno et al. 2016), whereas Kao et al. were the only ones to detect a lower mean copy number in asthenozoospermic samples than in normozoospermia samples (Kao et al. 2004).

In 2022, Shi show in his study that the increased sperm mitochondrial DNA copy number and DNA fragmentation index (DFI) contributed to poor seminal quality, although sperm mtDNA-CN, ROS, and DFI were not linked to assisted reproductive technology (ART) clinical outcomes.

MATERIAL AND METHODS

1. Study population and semen analysis

We selected 35 patients attending the Andrological Clinic of the Endocrinology Clinic - Department of Clinical and Molecular Sciences - University Hospital of Ancona. The exclusion criteria were the use of any medications (antibiotics, anabolic hormones) and/ or the presence of any medical conditions associated with impaired semen parameters (endocrine diseases, urogenital tract infections, varicocele, cryptorchidism, testicular or other cancer, previous chemotherapy and/or radiotherapy). Semen samples with leukocytospermia and/or increased viscosity were also excluded from this study. Written informed consent was obtained from all study participants. The semen examination was carried out in accordance with WHO 2010. The samples were obtained by masturbation after 3–5 days' abstinence. The following variables were taken into consideration: ejaculate volume (mL), pH, sperm concentration (N × 10^6 /mL), total sperm number (N × 10^6 /ejaculate), progressive motility (%) and morphology (% abnormal forms). A sperm viability test was carried out to differentiate cell death from immobility by staining with eosin Y 0.5% in saline solution.

Semen samples were divided into 3 groups according to their progressive motility and percentage of normal form as follow:

- *control group (CTRL, n=15)*: progressive motility > 32%, normal form > 4%).
- *asthenozoospermia group (AS, n=13)*: progressive motility < 32%.
- *asthenoteratozoospermia group (AST, n=7)*: progressive motility < 32%, normal form < 4%).

2. RNA Extraction

Semen samples were diluted with PBS to around 10 × 10^6 sperm/mL and underwent osmotic shock to eliminate the no gamete cell component. Aliquots of 1 ml of the diluted samples were centrifuged at 1600 g 10 min, and the pellets were incubated with 1 ml of cell lysis buffer (0.1% SDS, 0.5% Triton X-100 in distilled H_2O) for 60 min on ice. After incubation, the absence of any round cells was confirmed under the optical microscope.

Total RNA was extracted from ~10 × 10^6 sperm with TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) with small modification. Briefly, we resuspended the pellet in 250 µL of PBS without Ca2+/Mg2+, we added 750 µL of trizol, and we used a 26-gauge syringe for mechanical lysis. We incubate the mixture for 10 min at 37°C, then we added 200 µL of chloroform and proceeded according to the manufacturer's instructions RNA concentration and purity were quantified using NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was stored at −80 °C until use.

3. Quantitative RT-PCR and Analysis of the expression of TFAM and GPX

cDNA was synthesized from 50 ng of total RNA using the All-In-One 5X RT MasterMix (ABM, Richmond, BC, Canada). The reaction, with a final volume of 20µL, was incubated in "MASTER cycle realplex ep gradient" at 37°C for 15 min, 60°C for 10 min and 95°C for 3 min.

Quantitative real-time PCR was carried out using Step One Real-Time PCR System (Applied Biosystems), with the primers and fluorescent probes specific for PRM1, PRM2, TNP1 and TNP2 and with GAPDH as the endogenous control. Data were analyzed by ΔCt method.

Quantitative real-time PCR was performed using Master Cycle (Eppendorf, Hamburg, Germany) with FastStart SYBR Green Master (Roche, Basel, Switzerland), according to the manufacturer's instructions with the following PCR condition: denaturation of 95°C for 10 min followed by 40 denaturation cycles of 95°C for 15 sec and 60°C for 1 min. A melting stage (72°C for 60 sec) was added at the end of the amplification procedure. There was no non-specific amplification as determined by the melting curve.

Each sample of template cDNA was amplified at the same time in three separate PCRs for the three genes. The primer sequences were as follow:

- GAPDH_F:5'-AGCCACATCGCTCAGACAC-3'
  GAPDH_R: 5'-GCCCAATACGACCAAATCC-3'
- TFAM_F: 5'-GGCAAGTTGTCCAAAGAAACC-3'
  TFAM_R:5'-GCATCTGGGTTCTGAGCTTTA-3'
- GPx-1_F:5'-GTGCTCGGCTTCCCGTGCAAC-3'
  GPx-1_R-CTCGAAGAGCATGAAGTTGGGC

Each sample was loaded in triplicate onto the reaction plate, fluorescence data were converted to cycle threshold (Ct) by Expression Suite v1.0.4 (master cycle ep Replex) and the mean Ct value was considered for each gene. The reference gene GAPDH was used for normalization. The mRNA expression levels were analyzed with the $2^{-\Delta Ct}$ method. The values of the relative expression of genes of interest are given as mean ± SD, over three independent experiments.

4. Statistical analysis

Continuous variables are presented as mean, SD and median. The comparison between groups was carried out by T-test or Mann-Whitney test for independent samples and one-way ANOVA or Kruskal-Wallis's test, depending on the shape of distribution and the number of groups evaluated. For dependent samples, the ANOVA test for repeated measurements and the Friedman test were used, depending on the normal distribution. A two-tailed p value < 0.05 was considered as statistically significant. Statistical analysis was performed using SPSS 23 (SPSS Inc., Chicago, IL, USA).

RESULTS

❖ Semen characteristics

Thirty-five people made up the study population, of whom 13 had asthenozoospermia, seven had asthenoteratozoospermia, and fifteen had normozoospermia. Semen analysis was done on everyone.

Table 3 presents the means, standard deviations, and medians for the age and semen parameters of everyone.

The average age of infertile patients did not differ significantly from the control groups.

Progressive motilities were reduced between AS and AST vs CTRL (17.3 ±7.5% and 13.6 ± 3.7% vs 40.0 ± 9.4%, respectively; $p<0.001$) as well as total motility (27.6 ± 11.1% and 19.3 ± 6.7% vs 51.3 ± 7.2%, respectively; $p<0.001$). The percentage of the spermatozoa with abnormal forms was higher in AS and AST than in controls (92.0 ± 2.4% and 96.7 ± 0.5% vs 81.3 ± 4.4%, respectively; $p<0.001$). Lastly, the percentage of viability was significantly increased for asthenozoospermia and asthenoteratozoospermia groups compared to controls (31.8 ± 13.1% and 38.8 ± 18.7% vs 63.3 ± 14.9%, respectively; $p<0.001$).

❖ Comparisons of TFAM and GPX1 expression and their relationships to semen characteristics

Asthenozoospermia and asthenoteratozoospermia were compared to controls in terms of the spermatozoa's mitochondrial transcription factor, A (TFAM), and glutathione peroxidase (GPx-1) gene mRNA levels.

We found that TFAM gene expression was elevated and statistically significant in the asthenoteratozoospermia group (fold expression=1.30, $p=0.05$) and it was considerably downregulated in the asthenozoospermia group compared to controls (fold expression=1.86, $p=0.05$). **Figure 7**

We discovered a statistically significant downregulation of the TFAM gene in the asthenozoospermic compared to asthenoteratozoospermic groups (fold expression = 2.40, $p = 0.011$).

In addition, there were no apparent variations in GPx-1 gene expression across the three groups, and in both asthenoteratozoospermic and asthenozoospermic groups showed a downregulation and lower expression relative to control.

The analysis revealed, for the control group , a statistically significant positive correlations were found between semen sperm parameter; total sperm concentration (10^6/ejaculated), total sperm Motility, and viability (respectively, r=0.561, p=0.03, r=0.641, p=0.01), and between progressive motility, total motility , and viability (respectively; r=0.894, p=0.001, r=0.722, p=0.002), also a strong significant positive correlation between total motility, progressive motility, and, viability (respectively ; r=0.894, p=0.0001, r=0.835, p=0.001), and a significant positive correlation between TFAM gene expression and GPx-1 gene expression (r=0.639, p=0.01). However, we found a significant negative correlation between total sperm concentration (10^6/ejaculated) and abnormal form (AF) (r=-0.774, p=0.01), leucocyte concentration and viability (r=-0.612, p=0.01), and between progressive motility and abnormal form (AF) (r=-0.666, p=0.007), and total motility and abnormal form (AF) (r=-0.599, p=0.01).

On the other hand, for the asthenozoospermic (AS) group, a statistically significant positive correlation were found in semen sperm parameter; firstly, between progressive motility and total motility and viability (respectively; r=0.894, p=0.001, r=0.814, p=0.001), secondly between leucocyte sperm concentration, progressive motility, total motility and viability (respectively; r=0.576, p=0.003, r=0.735, p=0.004, r=0.671, p=0.001), thirdly, a significant positive correlation between total sperm motility and viability(r=0.800, p=0.001).

Furthermore, for the asthenoteratozoospermic (AST) group, no statistically significant correlation was found in semen sperm parameter, but we found a strong positive correlation between TFAM and GPx-1 gene expression (r=0.947, p=0.001) **Figure 8**

Table 3: Mean. SD and median (in brackets) for age and semen parameters of normozoospermic (CTRL). asthenozoospermia (AS) and asthenoteratozoospermia (AST)

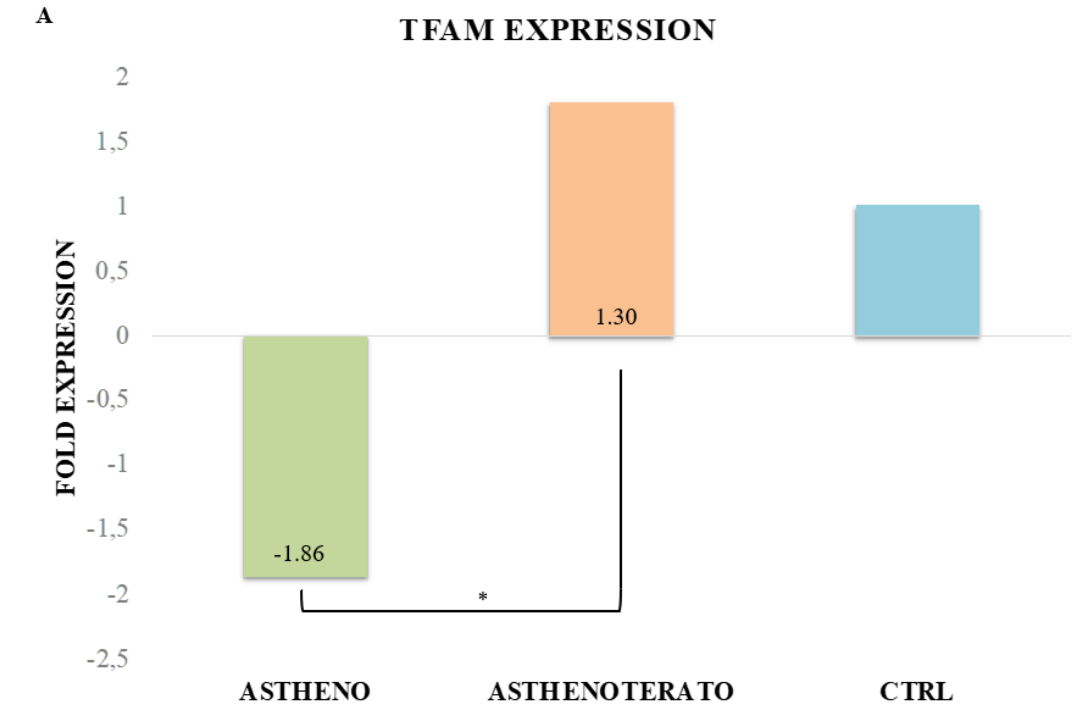
	Age (Years)	BMI (kg/m2)	Volume (ml)	PH	Total sperm number ($x10^6$/ejaculate)	Leucocyte ($\times10^6$/ ml)	Progressive Motility (%)	Total Motility (%)	Viability (%)	Abnormal F (%)
CRTL (n=15)	36.9±8.1 (37)	26.28±3.82 (26.37)	3.4±1.4 (3.10)	7.5±0.1 (7.50)	365.1±359.5 (279.5)	0.5±0.2 (0.50)	40.0 ±9.4 (40)	51.3±7.2 (50)	63.3±14.9 (64)	81.3±4.4 (82)
AS (n=13)	36.9±8.4 (36)	23.52±2.92 (23.63)	3.90±1.9 (3.60)	7.5±0.1 (7.50)	335.1 ±288.3 (235.6)	0.5±0.4 (0.30)	17.3±7.5 (15)	27.6±11.1 (25)	31.8±13.1 (29)	92.0±2.4 (72)
AST (n=7)	34.6±9.7 (40)	25.40±3.32 (24.84)	4.12±2.4 (3.10)	7.5±0.1 (7.60)	261.1±156.0 (235.2)	0.7±0.4 (0.80)	13.6±3.7 (15)	19.3±6.7 (20)	38.8±18.7 (32)	96.7±0. (97)
p-value[1]	*Ns*	*ns*	*ns*	*Ns*	*Ns*	*Ns*	*ns*	0.05	*Ns*	0.04
p-value[2]	*Ns*	*ns*	*ns*	*Ns*	*Ns*	*Ns*	*ns*	0.05	*Ns*	0.04
p-value[3]	*Ns*	*ns*	*ns*	*Ns*	*Ns*	*Ns*	<0.001	<0.001	<0.001	<0.001
p-value[4]	*Ns*	*ns*	*ns*	*Ns*	*Ns*	*Ns*	<0.0001	<0.001	<0.001	<0.001

p-value[1]: CRTL vs AS; *p-value[2]*: CRTL vs AST; *p-value[3]*: AS vs AST; *p-value[4]*: comparison between 3 groups

ns: p-value not significant.

*Significant difference $p \leq 0.05$; ** Significant difference $p \leq 0.001$

Figure 7. Relative expression of mRNA TFAM (A) and GPx (B) in ASTENO and ASTHENOTERATO patients vs. CTRL subjects, assuming normozoospermic (CTRL) = 1



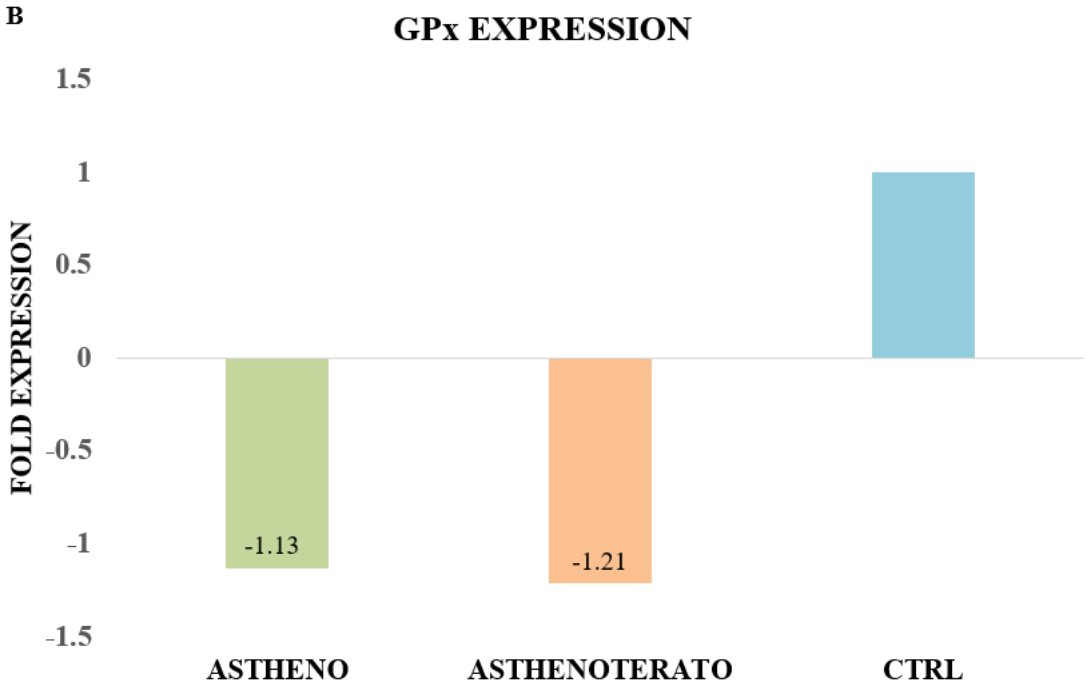
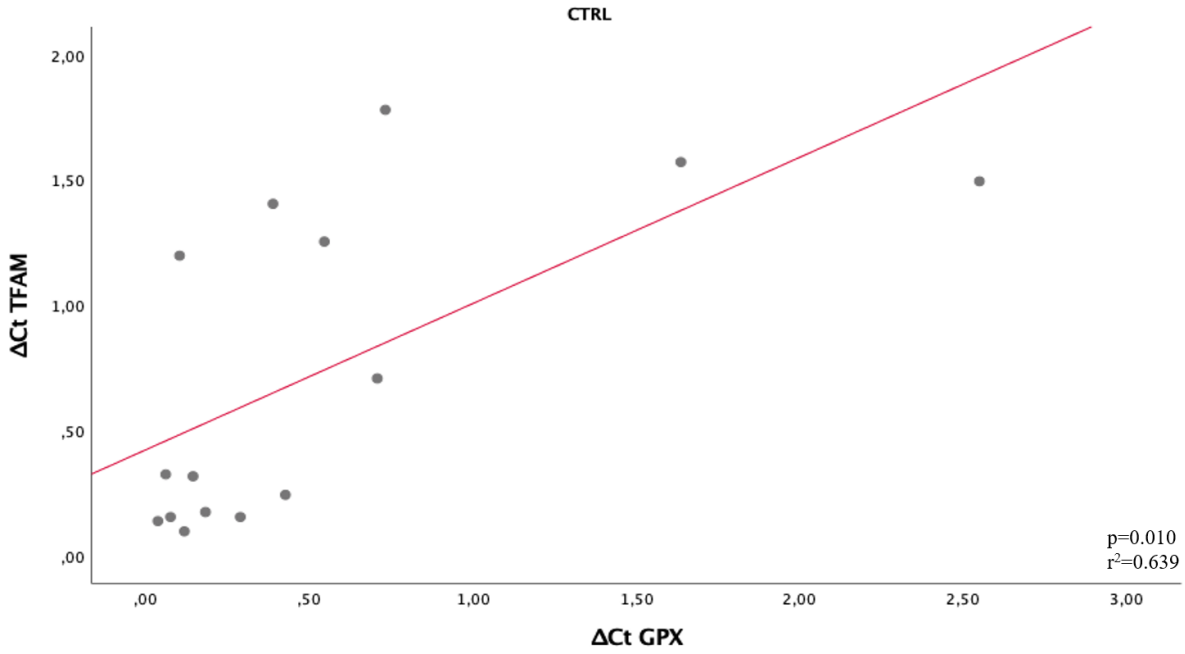
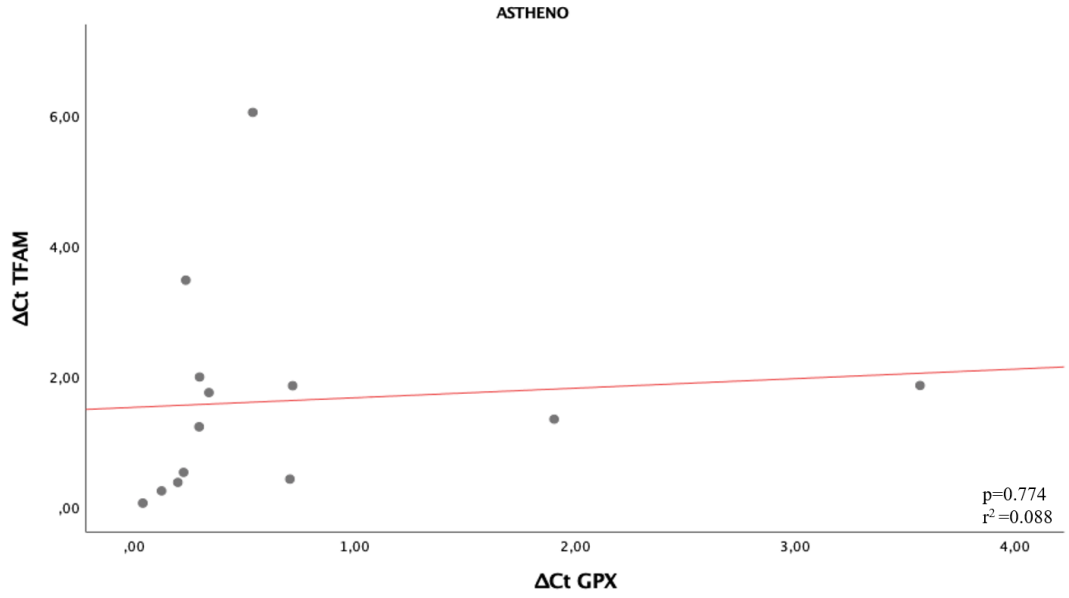


Figure 8: histograms of expression genes TFAM and GPx-1 correlation in the 3 studied groups, respectively controls(A), Asthenozoospermia(B), Asthenoteratozoospermia(C).

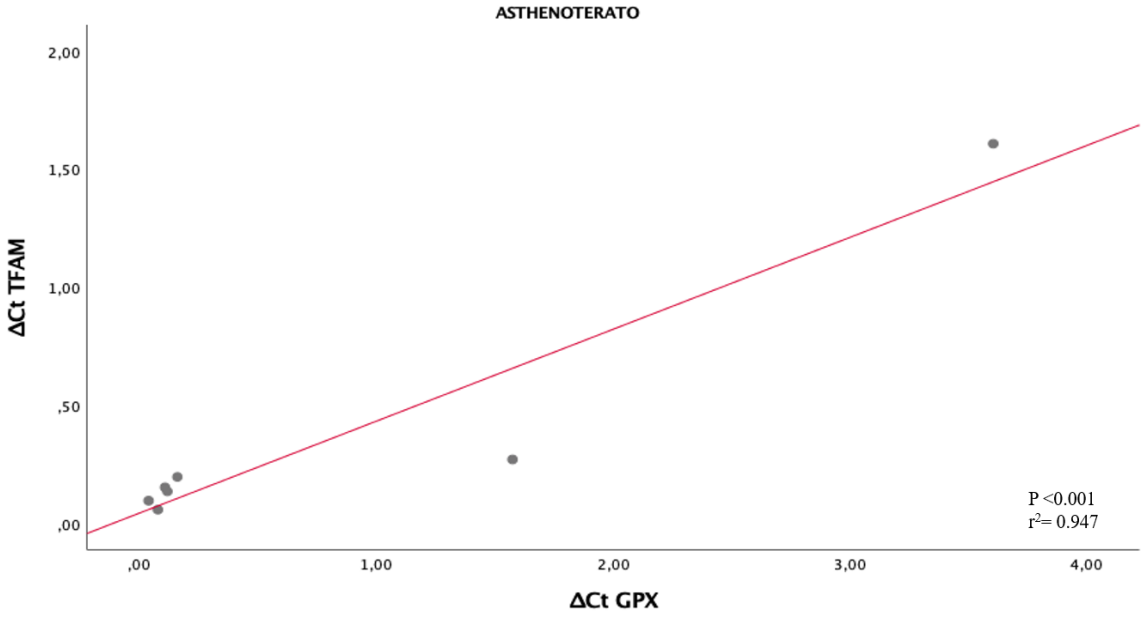
(A)



(B)



(C)


ASTHENOTERATO
2,00
1,50
1,00
,50
,00
ΔCt TFAM
,00
1,00
2,00
3,00
4,00
ΔCt GPX
P <0.001
r2= 0.947

DISCUSSION

The term "normal spermogram," based on statistical data from various studies of fertile men, only provides relative information; each parameter, when it is below the threshold of normality, constitutes an index for characterizing the pathology and his severity. Sperm is only fertilizing if it produces an embryo (Barriere et al. 1989).

First, we investigated roughly 35 Italian patients whose sperm count is larger than 15 million spermatozoa/ml for the first time whose mobility and/or morphology are aberrant, or the two parameters are altered, or whose morphology is normal, but their motility is abnormal.

The percentage of progressive mobility and total spermatozoa motility, as well as for vitality and the percentage of abnormal forms, were significantly different between asthenozoopsermia (AS) and astheno-teratozoospermia (AST) in the global ANOVA statistical study and other statistical tests of the sperm parameters. Age, seminal volume, sperm number, leucocyte number, and BMI did not significantly differ between groups, though.

It then becomes a matter of researching any potential link.

• Infertility patients' sperm parameters.

• Sperm parameter, mitochondrial gene expression; TFAM gene expression

• Sperm oxidative profile and sperm parameters; GPx-1gene expression.

The significance of sperm morphology in understanding male infertility is demonstrated by our results, which reveal a relationship between morphology and other sperm characteristics including motility and vitality. The preliminary findings of this investigation support the notion that sperm motility, morphology, and account may all be used to help diagnose disease (Guzick et al. 2001). Sperm morphology appears to be a more significant factor in male infertility than sperm count. The morphological polymorphism of spermatozoa is so extensive in man that he is essentially the only creature having it (Buffone et al. 2004, Chocat et al. 2001). Most experts now concur that the spermatozoa's morphology and mobility are the most aggravating factors that might affect male infertility (Marmor et al. 1990).

Age has no bearing on any of the sperm parameters in our study. Additionally, BMI did not exhibit any link with the sperm parameter, and this result is consistent with Puri et al. 2020 (Puri and al. 2020).

In humans, there is a huge range in the nuclear maturity of the sperm population, making it extremely diverse. The compaction of the DNA in the spermatozoa's nucleus is the first characteristic that distinguishes teratozoopsermia from normal sperm, according to research. This nuclear alteration may be the result of a defect in the decondensation of DNA during fertilization, leading to degeneration or poor development of the embryo. (Irvine et al. 2001). Nitric oxide (NO) content in the seminal fluid has been theorized to be the cause of the decreased sperm motility, concentration, and deformity (Mehraban et al. 2005, Romeo et al. 2003). In fact, the effect of NO on sperm will result in a decrease in the amount of ATP in the cells by inhibiting the enzyme ATP synthase, which is responsible for the loss of energy and, as a result, a change in motility (Kisa et al. 2004). The reactive oxygen species (ROS) molecules, which also include ions and hydrogen peroxide, include NO. ROS and DNA degradation are more likely to affect sperm with poor chromatin condensation (Pasqualotto et al. 2001). Moreover, the lack of repair enzymes results in the absence of repair mechanisms in spermatozoa. In contrast to normal sperm, alterated sperm is therefore thought to be a major source of free radicals like ROS and DNA denaturation (Kalthur et al. 2008, Ruiz-Pesini et al. 2000).

Previous studies have shown that ejaculated sperm from patients with a high level of morphologically abnormal spermatozoa and poor motility exhibits evidence of DNA damage, disturbed meiotic segregation, and oxidative stress induction characterized by impaired seminal antioxidant status and a high level of seminal lipid peroxidation (Brahem et al. 2011, Ammar et al. 2018, Aitken et al. 2020). Hence, decreased GPX levels in the seminal plasma may lead to excessive amounts of hydrogen peroxide and oxidative stress. What's more noteworthy is that hydrogen peroxide has been found to be the major ROS responsible for the motility impairment (Baumber et al. 2000, Atig et al. 2017). Crisol et al. also discovered decreased GPX activity in samples with severe asthenozoospermia, oligozoospermia, and teratozoospermia in comparison to normal (Crisol et al. 2012). Additionally, it has been noted that sperm with significantly worse sperm morphology had lower GSH levels inside their cells (Garrido et al. 2004) On the other side, Macanovic and associates discovered a negative correlation between GPX

activity and sperm morphology and motility. In addition, the evaluation of complete GPX rather than simply the most crucial isoform 1 alone (Macanovic et al. 2015).

Our research revealed that the GPX1 gene is downregulated on AS and AST compared to controls, but this finding is not statistically significant. These findings are in line with those reported by Garrido et al. in 2004, who also discovered no statistically significant difference. However, our results showed that GPX1 gene expression were lower in the seminal fluid of fertile individuals compared to infertile men and its levels were not significantly associated with sperm parameters, particularly motility and morphology. This result agrees with Garrido et al. (Garrido et al. in 2004). Alvarez et al. (1987; 1989) show that the rapid loss of motility in human spermatozoa, brought about by inactivation of spermatozoa.

The functioning of cells depends heavily on mitochondria, which are intracellular organelles. They perform a wide range of biological functions, including the synthesis of steroid hormones, ion homeostasis, apoptosis, and reactive oxygen signaling, in addition to producing energy. These organelles assist the sperm function and travel during the energy- and time-consuming process of spermatogenesis by forming tight helices at the sperm's core (Markaki, et al. 2020, Rosati et al. 2020, Valenti et al. 2021). Additionally, mtDNA is more vulnerable to oxidative stress (OS) than nuclear DNA because it is not compactly packed like nuclear DNA (Kumar et al. 2009). To perform their physiological duties, human spermatozoa produce ROS at low levels.

However, OS, which is marked by mitochondrial and nuclear DNA damage, could develop if the creation of these reactive free radicals overwhelms the antioxidant defense system (Venkatesh et al. 2009). Numerous research supports the association between male infertility and spermatozoa with mitochondrial abnormalities (Ankel-Simons et al. 1996, Hirata et al. 2002).

Sperm mitochondrial loss is a sign of both damaged and intact mtDNA. Sperm mitochondrial DNA deletions are more common in men with poor semen parameters than in men who are normozoospermic (Ieremiadou & Rodakis; 2009, Song & Lewis, 2008). While multiple mtDNA deletions are present in both testicular and ejaculated spermatozoa from fertile and infertile men, Kao proposed that mtDNA content may be an important indicator of sperm quality in 2004. Large-scale deletions of mtDNA were identified as risk factors for poor sperm quality in asthenoteratozoospermia-induced

male infertility (Kao et al. 2004, Gholinezhad et al. 2018). According to Song and Lewis, the spermatozoa had a considerable increase in mtDNA copy number and a loss in mtDNA integrity. Therefore, increased sperm mtDNAcn found in infertile males may be caused by abnormal DNA methylation, TFAM and POLG gene expression, or both. As an alternative, oxidative stress has been connected to sperm mtDNAcn and mtDNAdel as well as sperm characteristics (Abasalt et al. 2013; Bonanno et al., 2016, Agarwal et al., 2014; Aitken et al., 2014, Durairajanayagam et al 2021).

Uncertain biological links exist between mtDNA measurements, semen characteristics, and infertility. In our work, we evaluated Mitochondrial Transcription Factor A (TFAM) on the same patients concurrently with the investigation of the sperm characteristics, and as a result, we tracked the expression differences between groups. Mitochondrial Transcription Factor A (TFAM), which is expressed up until the late spermatocyte and early spermatid stage, oversees maintaining the integrity and transcriptional regulation of mitochondrial DNA (mtDNA) within the mitochondrial nucleoid. In the human testis, TFAM mRNA is not differently spliced, but its expression is downregulated (Larsson et al. 1997b). Additionally, TFAM reduction resulted in a 30% reduction in COX-1 expression, which killed embryos (Ekstrand et al. 2004). The cytochrome c oxidase has three subunits, three of which are encoded by the mitochondrial genome (Tsukihara et al. 1995).

The question is what molecular mechanisms and pathways can be involved to explain this absence of fertilization and to better understand the biological phenomenon involved in the spermatozoa of the astenoteratozoospermic and asthenozoospermic men we analysis the expression TFAM gene.

Since TFAM promotes the creation of the primer needed for mtDNA replication, it is believed that TFAM regulates the quantity of mtDNA copies (Ekstrand et al. 2004). To that end, multiple investigations demonstrate a potent relationship between mtDNA and TFAM levels (Poulton et al. 1994; Davis et al. 1996; Larsson et al. 1994; Seidel-Rogol and Shadel; 2002). In human testicular sperm, TFAM is only down-regulated (Larsson et al. 1997), which most likely corresponds with the existence of fewer copies of mtDNA. According to Amaral et al., superior sperm samples with lower mtDNA concentrations have more TFAM, which is the opposite of what occurs in other cell types (Zhao et al. 2006). Faja et al. found no statistically significant correlations

between the mtDNA copy number and semen parameters, a statistically significant negative correlation between the TFAM gene expression and the number of motile sperm per ejaculate, and a statistically significant positive correlation between the TFAM expression and the percentage of abnormal forms; however, we did not find any correlation in our results. According to the findings of our investigation, which are consistent with previous research, TFAM expression is significantly downregulated in the AST group.

Because it carries the paternal mitochondrial genome, TFAM protein is expected to be present in the mitochondrial sheath (Sutovsky and Schatten; 2000). Only being present in this non-mitochondrial region suggests that TFAM may be involved in infertility in another way. Contrarily, in our study, we did not find any correlation between TFAM and sperm parameter in infertile patient's vs control groups. Instead, previous studies demonstrated that the integrity and copy number of mtDNA were significantly correlated with sperm count (Cao et al. 2006, Escalier; 2006, and Kim et al. 2007). Moreover, Low-quality sperm have lower levels of TFAM. Higher sperm mtDNAcn in infertile men may reflect abnormalities during spermatogenesis such as aberrant gene expression of TFAM and POLG, which are known regulators of mtDNAc (Amaral et al. 2007).

These results are discrepant with those reported by Wu et al. who found that both sperm mtDNAcn and mtDNAdel were associated with lower sperm concentration, count, motility, and morphology. Additionally, we did not find a correlation between TFAM gene expression and the percentage of abnormal form, the percentage of progressive, or the percentage of total motility (Wu et al. 2019). MtDNA copy number is raised because of TFAM overexpression, but mitochondrial mass and respiratory chain capacity are not enhanced (Ekstrand et al., 2004).

The major finding in our study was the discovery of a positive correlation between TFAM and GPX expression; however, we did not find a significant difference in the expression of the other sperm parameters or genes. This is due, in part, to the small sample size, the heterogeneity of the patient population, and the inclusion of patients who were normozoospermic but sought fertility treatment using reproductive technology.

We need to expand our study population in the future and try to corroborate our findings with isolate population.

Conclusion

Our findings support the notion that oxidative stress and the mitochondria play a significant role in asthenozoospermia and asthenoteratozoospermia. Increased mitochondrial genome content may be able to make up for functional deficits caused by various etiologies. The TFAM gene expression and GPx-1 gene correlation, however, could be a sign of high-quality sperm and a sign of spermatogenetic dysfunction in infertile individuals because of their tight association with sperm motility and aberrant forms. These factors should be carefully considered, especially considering assisted reproductive technologies. The further examination of the molecular pathways in male infertility, particularly astenozoospermia and teratozoospermia, will be made possible by the future study of TFAM and the other major regulator of oxidative stress.

In Prospective.

Given the importance of these results, it would be interesting to continue the research, taking into consideration the following recommendations.

- To better understand the pathophysiology of sperm abnormalities, its necessary to study the structural and molecular modification of different components of spermatogenesis.
- Knowledge of the genetic causes and the discovery of new genes and new pathway involved in male infertility are essential for better understanding of the complex physiopathology of infertility such as Interleukin 8.

ARTICLES PUBLISHED

Attia H, Finocchi F, Orciani M, Mehdi M, Zidi Jrah I, Lazzarini R, Balercia G, Mattioli Belmonte M. Pro-inflammatory cytokines and microRNAs in male infertility. Mol Biol Rep. 2021 Aug;48(8):5935-5942. doi: 10.1007/s11033-021-06593-6. Epub 2021 Jul 28. PMID: 34319544; PMCID: PMC8376712.

https://pubmed.ncbi.nlm.nih.gov/34319544/

Reference

Abasalt HC, Gholamali JS, Maryam GC (2013). "Lipid peroxidation and large-scale deletions of mitochondrial DNA in asthenoteratozoospermic patients." Indian J Biochem Biophys 50(6): 492– 499.

Agarwal A, Mulgund A, Hamada A & Chyatte MR (2015) A unique view on male infertility around the globe. *Reproductive Biology and Endocrinology* 13 37.

Agarwal A, Maldonado Rosas I, Anagnostopoulou C, Cannarella R, Boitrelle F, Munoz LV, Finelli R,.

Agarwal A, Tvrda E, Sharma R. (2014) Relationship amongst teratozoospermia, seminal oxidative stress and male infertility. Reprod Biol Endocrinol.; 12:2–9.

Agarwal A, Virk G, Ong C, du Plessis SS (2014). "Effect of oxidative stress on male reproduction." World J Mens Health 32(1): 1–17.

Agarwal, A.; Sengupta, P. (2020). Oxidative stress, and its association with male infertility. In *Male Infertility*; Springer: Berlin/Heidelberg, Germany, pp. 57–68.

Afzelius BA, Eliasson R, Johnsen O, Lindholmer C.(1975) Lack of dynein arms in immotile human spermatozoa. J Cell Biol. Aug;66(2):225-32. doi: 10.1083/jcb.66.2.225.

Alsalman, A. R. S., Almashhedy, L. A., and Hadwan, M. H. (2018). Effect of Oral Zinc Supplementation on the Thiol Oxido-Reductive index and Thiol-Related Enzymes in Seminal Plasma and Spermatozoa of Iraqi Asthenospermic Patients. Biol. Trace Elem. Res. 184, 340–349.

Amorim. A., Fernandes T., Taveira N. (2019). Mitochondrial DNA in human identification: a review. PeerJ 7: e7314.

Aitken RJ, Smith TB, Jobling MS, Baker MA, De Iuliis GN (2014). "Oxidative stress and male reproductive health." Asian J Androl 16(1): 31–38.

Aitken RJ, Krausz C. (2001). Oxidative stress, DNA damage and the Y chromosome. Reproduction. Oct;122(4):497-506.

Aitken R John. (2020) Impact of oxidative stress on male and female germ cells: implications for fertility review. Reproduction 159 R189–R201.

Alahmar, A.T.; Calogero, A.E.; Sengupta, P.; Dutta, S. (2021). Coenzyme q10 improves sperm parameters, oxidative stress markers and sperm DNA fragmentation in infertile patients with idiopathic oligoasthenozoospermia. *World J. Men's Health*, *39*, 346–351.

Alvarez JG, Touchstone JC, Blasco L, Storey BT (1987): Spontaneous lipid peroxidation and prcduction of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. J Androl8:338-348.

Alvarez, J. G., and B. T. Storey (1989). Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. Gamete Res. 2:77–90.

Amaral A, Joao Ramalho-Santos2 and Justin C.St John. (2007). The expression of polymerase gamma and mitochondrial transcription factor A and the regulation of mitochondrial DNA content in mature human sperm. Human Reproduction Vol.22, No.6 pp. 1585–1596, 2007

Amaral A, Lourenco B, Marques M & Ramalho-Santos J (2013). Mitochondria functionality and sperm quality. *Reproduction* 146 R163–R174.

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA & Sanger F et al. (1981). Sequence and organization of the human mitochondrial genome. *Nature* 290 457–465.

Ankel-Simons, F.; Cummins, J.M. (1996). Misconceptions about mitochondria and mammalian fertilization: Implications for theories on human evolution. Proc. Natl. Acad. Sci. USA, 93, 13859–13863.

Atig F, Kerkeni A, Saad A, Ajina M. (2017). Effects of reduced seminal enzymatic antioxidants on sperm DNA fragmentation and semen quality of Tunisian infertile men. J Assist Reprod Genet; 34:373–381

Ammar Oumaima, Tesnim Ajina, Zohra Haouas, Amira Sallem, Ines Zidi, Sana Chakroun, Intissar Grissa, Lobna Ezzi, Ali Jlali, Meriem Mehdi (2018). Investigation on the origin of sperm morphological defects: oxidative attacks, chromatin immaturity, and DNA fragmentation. Environmental Science and Pollution Research.;25(14):13775–13786

Busetto GM, Agarwal A, Virmani A, Antonini G, Ragonesi G, Del Giudice F, Micic S, Gentile V, De Browne RW, Bloom MS, Schisterman EF, Hovey K, Trevisan M, Wu C, Liu A, Wactawski-Wende J. (2008). Analytical and biological variation of biomarkers of oxidative stress during the menstrual cycle. Biomarkers. Mar;13(2):160-83.

Baumber J, Ball BA, gravance CG, Medina V, Davies-Morel MC (2000) The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J Androl 21(6):895–902.

Buffone MG, Doncel GF, Marin Briggiler CI, Vazquez-Levin MH, Calamera JC. (2004). Human sperm subpopulations: relationship between functional quality and protein tyrosine phosphorylation. Hum Reprod; 19:139–146.

Bibb MJ, Van Etten RA, Wright CT, Walberg MW & Clayton DA (1981). Sequence, and gene organization of mouse mitochondrial DNA. *Cell* 26 167–180.
Bogenhagen D & Clayton DA (1977). Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. *Cell* 11 719–727.
Brigelius-Flohé R, Maiorino M. (2013). Glutathione peroxidases. Biochim Biophys Acta. May; 1830(5): 3289-303.
Bonanno O, Romeo G, Asero P, Pezzino FM, Castiglione R, Burrello N, Sidoti G, Frajese GV, Vicari E, D'Agata R (2016). "Sperm of patients with severe asthenozoospermia show biochemical, molecular and genomic alterations." Reproduction 152(6): 695–704.
Brahem S, Mehdi M, Elghezal H, Saad A. (2011) Detection of DNA fragmentation and meiotic segregation in human with isolated teratozoospermia. J Assist Reprod Genet; 28:41–48.
Campbell CT, Kolesar JE, Kaufman BA. (2012). Mitochondrial transcription factor A regulates mitochondrial transcription initiation, DNA packaging, and genome copy number. Biochim Biophys Acta. Sep-Oct;1819(9-10):921-9.
Cao, W., L. Haig-Ladewig, G. L. Gerton, and S. B. Moss.(2006). Adenylate kinases 1 and 2 are part of the accessory structures in the mouse sperm flagellum. Biol Reprod 75: 492-500.
Chimienti, A. Picca, G. Sirago, F. Fracasso, R. Calvani, R. Bernabei, F. Russo, C.S. Carter, C. Leeuwenburgh, V. Pesce, E. Marzetti, A.M.S. Lezza, (2018). Increased TFAM binding to mtDNA damage hot spots is associated with mtDNA loss in aged rat heart, Free Radic. Biol. Med. 124 447–453.
Colagar AH, Marzony ET, Chaichi MJ.(2009). Zinc levels in seminal plasma are associated with sperm quality in fertile and infertile men. Nutr Res. Feb;29(2):82-8.
Cardullo RA, Baltz JM. (1991). Metabolic regulation in mammalian sperm: mitochondrial volume determines sperm length and flagellar beat frequency. Cell Motil Cytoskeleton; 19:180–8.
Carra E, Sangiorgi D, Gattuccio F & Rinaldi AM (2004) Male infertility and mitochondrial DNA. *Biochemical and Biophysical Research Communications* 322 333–339.
Chada S. (2015) Post-transcriptional regulation of glutathione peroxidase gene expression by selenium in the HL-60 human myeloid cell line. Blood.;74(3):930-939
Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ, Carrell DT.(2006). Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. J Androl. Jan-Feb;27(1):53-9
Chacinska A, Koehler CM, Milenkovic D, Lithgow T & Pfanner N(2009) Importing mitochondrial proteins: machineries and mechanisms. *Cell* 138 628–644.
Chocat, A & Creveuil, C & Galeraud-Denis, Isabelie & Herlicoviez, D & Herlicoviez, Michel & Sauvalle, A. (2001). [Predictive value of non-automated spermatic parameters and automated kinetic parameters on cleavage rate in fertilization in vitro]. Gynécologie, obstétrique & fertilité. 29. 301-7.
Crisol L, Matorras R, Aspichueta F, Expósito A, Hernández ML, Ruiz-Larrea MB, Mendoza R, Ruiz-Sanz JI (2012) Glutathione peroxidase activity in seminal plasma and its relationship to classical sperm parameters and in vitro fertilization-intracytoplasmic sperm injection outcome. Fertil Steril 97(4):852–857 e1
Cummins JM, Jequier AM, Kan R. Molecular biology of human male infertility: links with aging, mitochondrial genetics, and oxidative stress? Mol Reprod Dev (1994); 37:345–62.
Davis AF, Ropp PA, Clayton DA and Copeland WC (1996) Mitochondrial DNA polymerase gamma is expressed and translated in the absence of mitochondrial DNA maintenance and replication. Nucleic Acids Res 24,2753–2759.
Dadoune JP, Mayaux MJ, Guihard-Moscato ML.(1988). Correlation between defects in chromatin condensation of human spermatozoa stained by aniline blue and semen characteristics. Andrologia. May-Jun;20(3):211-7.
Deponte M. (2013). Glutathione catalysis and the reaction mechanisms of glutathione-dependent enzymes. Biochim Biophys Acta. May;1830(5):3217-66.
De Braekeleer M, Nguyen MH, Morel F, Perrin A. (2015). Genetic aspects of monomorphic teratozoospermia: a review. J Assist Reprod Genet. Apr;32(4):615-23.
De Martino C, Floridi A, Marcante ML, Malorni W, Scorza Barcellona P, Bellocci M & Silvestrini B (1979). Morphological, histochemical, and biochemical studies on germ cell mitochondria of normal rats. *Cell and Tissue Research* 196 1–22.
de Rooij DG (2017). The nature and dynamics of spermatogonial stem cells. *Development* 144 3022–3030.
Demain LA, Conway GS & Newman WG (2017). Genetics of mitochondrial dysfunction and infertility. *Clinical Genetics* 91 199–207.
Durairajanayagam D, Singh D, Agarwal A, Henkel R. (2021). Causes and consequences of sperm mitochondrial dysfunction. Andrologia. Feb;53(1): e13666.

Dohle GR, Halley DJ, Van Hemel JO, van den Ouwel AM, Pieters MH, Weber RF, Govaerts LC. (2002) Genetic risk factors in infertile men with severe oligozoospermia and azoospermia. Hum Reprod. Jan;17(1):13-6.
Dorigo B, Schalch T, Kulangara A, Duda S, Schroeder RR, Richmond TJ. (2004). Nucleosome arrays reveal the two-start organization of the chromatin fiber. Science. Nov 26;306(5701):1571-3.
Evenson DP, Larson KL, Jost LK. (2002). Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl. Jan-Feb;23(1):25-43.
Ekoue DN, He C, Diamond AM, Bonini MG. (2017). Manganese superoxide dismutase and glutathione peroxidase-1 contribute to the rise and fall of mitochondrial reactive oxygen species which drive oncogenesis. Biochim Biophys Acta Bioenerg. Aug;1858(8):628-632.
Erden-Inal M, Sunal EKG. (2002). Age-related changes in the glutathione redox system. Cell Biochemistry and Function.20:61-66
Ekstrand MI, Falkenberg M, Rantanen A, Park CB, Gaspari M, Hultenby K, Rustin P, Gustafsson CM and Larsson NG (2004). Mitochondrial transcription factor A regulates mtDNA copy number in mammals. Hum Mol Genet 13,935–944
Escalier, D. (2006). Arrest of flagellum morphogenesis with fibrous sheath immaturity of human spermatozoa. Andrologia 38: 54-60.
Faja F, Tania Carlini, Giulia Coltrinari, Federica Finocchi,· Matteo Nespoli, Francesco Pallotti, Andrea Lenzi, Francesco Lombardo, · Donatella Paoli.(2019). Human sperm motility: a molecular study of mitochondrial DNA, mitochondrial transcription factor A gene and DNA fragmentation
Farhat Sarah, Florent Isabelle, Noel Benjamin, Kayal Ehsan, Da Silva Corinne, Bigeard Estelle, Alberti Adriana, Labadie Karine, Corre Erwan, Aury Jean-Marc, Rombauts Stephane, Wincker Patrick, Guillou Laure, Porcel Betina M. (2018). Comparative Time-Scale Gene Expression Analysis Highlights the Infection Processes of Two Amoebophrya Strains. Frontiers in Microbiology. VOL.9
Fainberg J, Kashanian JA. (2019). Recent advances in understanding and managing male infertility. F1000Res. May 16;8: F1000 Faculty Rev-670.
Foresta, C., Flohé, L., Garolla, A., Roveri, A., Ursini, F. & Maiorino, M. (2002). Male fertility and the selenoprotein PHGPx. Biology of Reproduction 67, 967–997.
Fernández JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. (2003). The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl. Jan-Feb;24(1):59-66
Fisher RP & Clayton DA (1988). Purification and characterization of human mitochondrial transcription factor 1. *Molecular and Cellular Biology* 8 3496–3509.
Folgero T, Bertheussen K, Lindal S, Torbergsen T & Oian P (1993). Mitochondrial disease, and reduced sperm motility. *Human Reproduction* 8 1863–1868.
Feng Q, Shao M, Han J, Tang T, Zhang Y, Liu F. (2020) TFAM, a potential oxidative stress biomarker used for monitoring environment pollutants in Musca domestica. Int J Biol Macromol. Jul 15;155: 524-534.
Irvin DK, Zurcher SD, Nguyen T, Weinmaster G, Kornblum HI. (2001). Expression patterns of Notch1, Notch2, and Notch3 suggest multiple functional roles for the Notch-DSL signaling system during brain development. J Comp Neurol. Jul 23;436(2):167-81.
Ieremiadou F, Rodakis GC. (2009). Correlation of the 4977 bp mitochondrial DNA deletion with human sperm dysfunction. BMC Res Notes. Feb 4; 2:18.
Garrido N, Meseguer M, Alvarez J, Simón C, Pellicer A, Remohí J (2004) Relationship among standard semen parameters, glutathione peroxidase/glutathione reductase activity, and mRNA expression and reduced glutathione content in ejaculated spermatozoa from fertile and infertile men. Fertil Steril 82:1059–1066.
Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, Carson SA, Cisneros P, Steinkampf MP, Hill JA, Xu D, Vogel DL. (2001). National Cooperative Reproductive Medicine Network. Sperm morphology, motility, and concentration in fertile and infertile men. N Engl J Med. Nov 8;345(19):1388-93.
Gholinezhad M, Yousefnia-Pasha Y, Hosseinzadeh Colagar A, Mohammadoo-Khorasani M, Bidmeshkipour A. (2018). Comparison of large-scale deletions of the sperm mitochondrial DNA in normozoospermic and asthenoteratozoospermic men. J Cell Biochem. Sep 11. doi: 10.1002/jcb.27492. Epub ahead of print.
Gholinezhad, M.; Yousefnia-Pasha, Y.; Colagar, A.H.; Mohammadoo-Khorasani, M.; Bidmeshkipour, A. (2018). Comparison of large-scale deletions of the sperm mitochondrial DNA in normozoospermic and asthenoteratozoospermic men. J. Cell. Biochem. 120, 1958–1968.

Gopalkrishnan K, Hinduja IN, Anand Kumar TC. (1991). Assessment of mitochondrial activity of human spermatozoa: motility/viability in fertile/infertile men. Mol Androl 3:243–50.
Gurung P, Yetiskul E, Jialal I. Physiology, Male Reproductive System. (https://www.ncbi.nlm.nih.gov /books/NBK538429/) StatPearls.
Hammadeh ME, Zavos PM, Rosenbaum P, Schmidt W. (2001). Comparison between the quality and function of sperm after semen processing with two different methods. Asian J Androl. Jun;3(2):125-30.
Hamada A, Sharma R, du Plessis SS, Willard B, Yadav SP, Sabanegh E & Agarwal A (2013). Two-dimensional differential in-gel electrophoresis-based proteomics of male gametes in relation to oxidative stress. *Fertility and Sterility* 99 1216. e2–1226.e2.
Hara K, Nakagawa T,Enomoto H, Suzuki M, Yamamoto M, Simons BD & Yoshida S (2014). Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. *Cell Stem Cell* 14 658–672.
Hatfield D. L., Tsuji P. A., Carlson B. A., Gladyshev V. N. (2014). Selenium and selenocysteine: roles in cancer, health, and development. *Trends Biochem. Sci*. 39, 112–120
Hess RA, Miller LA, Kirby JD, Margoliash E & Goldberg E (1993). Immunoelectron microscopic localization of testicular and somatic cytochromes c in the seminiferous epithelium of the rat. *Biology of Reproduction* 48 1299–1308.
Hirata, S.; Hoshi, K.; Shoda, T.; Mabuchi, T.(2002). Spermatozoon and mitochondrial DNA. Reprod. Med. Biol., 1, 41–47.
Huang C., Cao X., Pang D., Li C., Luo Q., Zou Y., Feng B., Li L., Cheng A., Chen Z. (2018). Is male infertility associated with increased oxidative stress in seminal plasma? A-meta-analysis. Oncotarget. 9: 24494-24513.
Imai, H. & Nakagawa, Y. (2003) Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. Free Radical Biology and Medicine 34, 145–169.
Jiang M, Kauppila TES, Motori E, Li X, Atanassov I, Folz-Donahue K, Bonekamp NA, Albarran-Gutierrez S, Stewart JB & Larsson NG. (2017). Increased total mtDNA copy number cures male infertility despite unaltered mtDNA mutation load. *Cell Metabolism* 26 429.e4–436.e4.
Johns DR. (1995). Mitochondrial DNA and disease. N Engl J Med; 333:638–44.
John ST, J. C., R. P. Jokhi, and C. L. Barratt. (2005a). The impact of mitochondrial genetics on male infertility. Int J Androl 28: 65-73
John JC, Cooke ID, Barratt CLR. (1997). Mitochondrial mutations and male infertility [Letter]. Nat Med .3:124–5.
Kang I, Chu CT, Kaufman BA. (2018). The mitochondrial transcription factor TFAM in neurodegeneration: emerging evidence and mechanisms. FEBS Lett. Mar;592(5):793-811.
Kanki T, Nakayama H, Sasaki N, Takio K, Alam TI, Hamasaki N & Kang D (2004). Mitochondrial nucleoid, and transcription factor A. *Annals of the New York Academy of Sciences* 1011 61–68.
Kao S, Chao HT & Wei YH (1995). Mitochondrial deoxyribonucleic acid 4977-bp deletion is associated with diminished fertility and motility of human sperm. *Biology of Reproduction* 52 729–736.
Kao Shu-Huei, Hsiang-Tai Chao, Hwan-Wun Liu, Tien-Lin Liao, and Yau-Huei Wei, (2004). Sperm mitochondrial DNA depletion in men with asthenospermia. fertility and sterility.vol. 82, no.1.
Kalthur, G., Adiga, S. K., Upadhya, D., Rao, S. and Kumar, P. (2008). Effect of cryopreservation on sperm DNA integrity in patients with teratospermia. Fertil Steril 89(6):1723–1727.
Kierszenbaum AL. (1994). Mammalian spermatogenesis in vivo and in vitro: a partnership of spermatogenic and somatic cell lineages. Endocr Rev 15: 116-34).
Kisa U, Başar MM, Ferhat M, Yilmaz E, Başar H, Cağlayan O, Batislam E. (2004). Testicular tissue nitric oxide and thiobarbituric acid reactive substance levels: evaluation with respect to the pathogenesis of varicocele. Urol Res. Jun;32(3):196-9.
Kim, Y. H. et al. (2007). Compartmentalization of a unique adp/atp carrier protein sfec (sperm flagellar energy carrier, aac4) with glycolytic enzymes in the fibrous sheath of the human sperm flagellar principal piece. Dev Biol 302: 463-476.
Kerr JB, Loveland KL, O'Bryan MK, Kretser DM. (2006). Cytology of the testis and intrinsic control mechanisms. In: Neill JD, ed. Physiology of Reproduction. Birmingham, AL: Elsevier; 827–947.
Kote-Jarai Z, Durocher F, Edwards SM, Hamoudi R, Jackson RA, Ardern-Jones A, Murkin A, Dearnaley DP, Kirby R, Houlston R, Easton DF, and Eeles R. (2002). Association between the GCG polymorphism of the selenium dependent GPX1 gene and the risk of young onset prostate cancer. Prostate Cancer Prostatic Dis 5: 189–192.
Kryukov G. V., Castellano S., Novoselov S. V., Lobanov A. V., Zehtab O., Guigó R., Gladyshev V. N. (2003). Characterization of mammalian selenoproteomes. Science 300, 1439–1443

Kumar R, Venkatesh S, Kumar M, Tanwar M, Shasmsi MB, Kumar R, Gupta NP, Sharma RK, Talwar P, Dada R. (2009). Oxidative stress and sperm mitochondrial DNA mutation in idiopathic oligoasthenozoospermic men. Indian J Biochem Biophys. Apr;46(2):172-7.
Larsson NG, Oldfors A, Garman JD, Barsh GS, Clayton DA. (1997). Downregulation of mitochondrial transcription factor A during spermatogenesis in humans. Hum Mol Genet. Feb;6(2):185-91.
Lavoie M-E, Lhoret RR, Ziai S, Lavoie J-C. (2011). Blood glutathione peroxidase activity in relation with the risk of cardiovascular diseases in obese women. Journal of Diabetes and Metabolism. 2(05):2-6
Lu J, Holmgren A. (2014). The thioredoxin antioxidant system. Free Radic Biol Med. Jan; 66:75-87.
Larsson NG, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, Barsh GS & Clayton DA (1998). Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nature Genetics* 18 231–236.
Liu Chunyu, Haruhiko Miyata, Yang Gao, Yanwei Sha, Shuyan Tang, Zoulan Xu, Marjorie Whitfield, Catherine Patrat, Huan Wu, Emmanuel Dulioust, Shixiong Tian, Keisuke Shimada, Jiangshan Cong, Taichi Noda, Hang Li, Akane Morohoshi, Caroline Cazin, Zine-Eddine Kherraf, Christophe Arnoult, Li Jin, Xiaojin He, Pierre F. Ray, Yunxia Cao, Aminata Touré, Feng Zhang, Masahito Ikawa,(2020). Bi-allelic DNAH8 Variants Lead to Multiple Morphological Abnormalities of the Sperm Flagella and Primary Male Infertility, The American Journal of Human Genetics, Volume 107, Issue 2.
Larsson, N. G., A. Oldfors, J. D. Garman, G. S. Barsh, and D. A. Clayton. (1997b). Downregulation of mitochondrial transcription factor a during spermatogenesis in humans. Hum Mol Genet 6: 185-191.
Li H, Wang J, Wilhelmsson H, Hansson A, Thoren P, Duffy J, Rustin P and Larsson NG (2000) Genetic modification of survival in tissue-specific knockout mice with mitochondrial cardiomyopathy. Proc Natl Acad Sci USA 97,3467–3472.
Methorst c., E. Huyghe and al. (2014). Stress oxydant et infertilité masculine: physiopathologie et intérêt thérapeutique des antioxydants. Urologie.24, 410.
Marmar JL. (2001). The diagnosis and treatment of male infertility in the new millennium. Int J Fertil Womens Med. May-Jun;46(3):116-36.
Marmor, D. bi Grob-Menendez, F. (1990) Les anomalies du flagelle dans le spermocytogramme. Journal de GynCcologie ObstCtrique et Biologie de la Reproduction, 19, 181- 185.
Macanovic B, Vucetic M, Jankovic A, Stancic A, Buzadzic B, Garalejic E, Korac A, Korac B, Otasevic V (2015) Correlation between sperm parameters and protein expression of antioxidative defense enzymes in seminal plasma: a pilot study. Dis Markers:1–5.
Mackenna A. (1995). Contribution of the male factor to unexplained infertility: a review. Int J Androl;18(Suppl 1):58–61.
Markaki, M.; Tavernarakis, N. (2020). Mitochondrial turnover and homeostasis in ageing and neurodegeneration. FEBS Lett. 594, 2370–2379.
Meinhardt A, Wilhelm B & Seitz J (1999) Expression of mitochondrial marker proteins during spermatogenesis. *Human Reproduction Update* 5 108–119.
Mehraban D, Ansari M, Keyhan H, Sedighi Gilani M, Naderi G, Esfehani F. (2005). Comparison of nitric oxide concentration in seminal fluid between infertile patients with and without varicocele and normal fertile men. Urol J. Spring;2(2):106-10.
Mirault ME, Tremblay A, Beaudoin N, and Tremblay M. (1991). Overexpression of seleno-glutathione peroxidase by gene transfer enhances the resistance of T47D human breast cells to clastogenic oxidants. J Biol Chem 266: 20752–20760.
Miranda-Vilela AL, Alves PC, Akimoto AK, Lordelo GS, Gonçalves CA, Grisolia CK, Klautau-Guimarães MN. (2010). Gene polymorphisms against DNA damage induced by hydrogen peroxide in leukocytes of healthy humans through comet assay: a quasi-experimental study. Environ Health. May 5; 9:21.
Mills, G. C. (1957). Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. J Biol Chem 229:189–97.
Mundy AJ, Ryder TA & Edmonds DK 1995 Asthenozoospermia and the human sperm mid-piece. *Human Reproduction* 10 116–119.
Morales-Alamo, D., and Calbet, J. A. L. (2014). Free radicals and sprint exercise in humans. Free Radic. Res. 48, 30–42.
Nguyen V.D., M.J. Saaranen, A.R. Karala , A.K. Lappi, L. Wang, I.B. Raykhel , H.I. Alanen, K.E. Salo , C.C. Wang, L.W. Ruddock. (2011). Two endoplasmic reticulum PDI peroxidases increase the efficiency of the use of peroxide during disulfide bond formation.J. Mol. Biol., 406, pp. 503-515
Nicolás Garrido, Ph.D., a,b Marcos Meseguer, Ph.D.,a,b Juan Alvarez, M.D.,a,c,d Carlos Simón, M.D.a,e Antonio Pellicer, M.D.,a,e,f and José Remohı´, M.D.a,e(2004). Relationship among standard semen parameters, glutathione peroxidase/glutathione reductase activity, and mRNA expression and reduced glutathione content in ejaculated spermatozoa from fertile and infertile men. fertility and sterility. vol. 82.

Otani H, Tanaka O, Kasai K & Yoshioka T (1988). Development of mitochondrial helical sheath in the middle piece of the mouse spermatid tail: regular dispositions and synchronized changes. *Anatomical Record* 222 26–33.
Pascual ML, Cebrian-Perez JA, Lopez-Perez MJ, Muino-Blanco T. (1996). Short-term inhibition of the energy metabolism affects motility but not surface properties of sperm cells. Biosci Rep, 16:35– 40. 13.
Halangk W, Bohnenback R, Kunz W. (1985). Interdependence of mitochondrial ATP production and extramitochondrial ATP utilization in intact spermatozoa. Biochim Biophys Acta 808:316–22.
Pasqualotto, F. F., Sharma, R. K., Kobayashi, H., Nelson, D. R., Thomas, A. J. Jr & Agarwal, A. (2001). Oxidative stress in normospermic men undergoing infertility evaluation. *Journal of Andrology*, **22**, 316–322.
Poulton J, Morten K, Freeman-Emmerson C, Potter C, Sewry C, Dubowitz V, Kidd H, Stephenson J, Whitehouse W, Hansen FJ et al. (1994). Deficiency of the human mitochondrial transcription factor h-mtTFA in infantile mitochondrial myopathy is associated with mtDNA depletion. Hum Mol Genet 3,1763–1769
Pournasir, M., Ghorbian, S., Ghasemnejad, T. et al. (2021). Glutathione peroxidase 3 (extracellular isoform) levels and functional polymorphisms in fertile and infertile men. Middle East Fertil Soc J 26, 13.
Puzio-Kuter AM. (2011). The role of p53 in metabolic regulation. Genes and Cancer. 2(4):385-391
Pastori, D., Pignatelli, P., Farcomeni, A., Menichelli, D., Nocella, C., Carnevale, R., & Violi, F. (2016). Aging-related decline of glutathione peroxidase 3 and risk of cardiovascular events in patients with atrial fibrillation. *Journal of the American Heart Association*, 5.
Powers SK, Duarte J, Kavazis AN, Talbert EE. (2010). Reactive oxygen species are signalling molecules for skeletal muscle adaptation. Exp Physiol. Jan;95(1):1-9.
Prete CD, Ciani F, Tafuri S, Pasolini MP, Valle GD, Palumbo V, Abbondante L, Calamo A, Barbato V, Gualtieri R, Talevi R, Cocchia N. (2018). Effect of superoxide dismutase, catalase, and glutathione peroxidase supplementation in the extender on chilled semen of fertile and hypofertile dogs. J Vet Sci. Sep 30;19(5):667-675.
Puri V, Sharma S, Gaur K, Sharma S, Shukla S. (2019). Association between male body mass index and semen parameters. Indian J Med Sci;71(3):109-12.
Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S, Dhama K. (2014). Oxidative stress, prooxidants, and antioxidants: the interplay. Biomed Res Int.2014:761264.
Rashki Ghaleno L, Alizadeh A, Drevet JR, Shahverdi A, Valojerdi MR. (2021). Oxidation of Sperm DNA and Male Infertility. Antioxidants (Basel). Jan 12;10(1):97.
Rengan, A.K., Agarwal, A., van der Linde, M. *et al.* (2012). An investigation of excess residual cytoplasm in human spermatozoa and its distinction from the cytoplasmic droplet. *Reprod Biol Endocrinol* 10, 92.
Rivlin J, Mendel J, Rubinstein S, Etkovitz N, Breitbart H. (2004). Role of hydrogen peroxide in sperm capacitation and acrosome reaction. Biol Reprod.Feb;70(2):518-22.
Romeo C, Ientile R, Santoro G, Impellizzeri P, Turiaco N, Impala P, Cifala S, Cutroneo G, Trimarchi F, Gentile C. (2001). Nitric oxide production is increased in the spermatic veins of adolescents with left idiophatic varicocele. J Pediatr Surg. 36: 389-93.
Roux C, Tripogney C, Joanne C, Bresson JL. (2004). Nuclear quality of the spermatozoon: exploration tests of the chromatin of human spermatozoa (nuclear proteins). Gynecologie, Obstetrique & Fertilite. Sep;32(9):792-798.
Reichert AS & Neupert W (2002). Contact sites between the outer and inner membrane of mitochondria-role in protein transport. *Biochimica et Biophysica Acta* 1592 41–49.
Rosati, A.J.; Whitcomb, B.W.; Brandon, N.; Louis, G.M.B.; Mumford, S.L.; Schisterman, E.F.; Pilsner, J.R. (2020). Sperm mitochondrial DNA biomarkers and couple fecundity. Hum. Reprod. 35, 2619–2625.
Ruiz-Pesini E, Diez C, Lapena AC, Perez-Martos A, Montoya J, Alvarez E, Arenas J & Lopez-Perez MJ (1998). Correlation of sperm motility with mitochondrial enzymatic activities. *Clinical Chemistry* 44 1616–1620.
Saleh Ramadan A, Ashok Agarwal, Essam A Nada, Mohamed H El-Tonsy, Rakesh K Sharma, Andrew Meyer, David R Nelson, Anthony J Thomas, (2022). Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility, Fertility and Sterility, Volume 79.
Shi Yonghong, Anke Dierckx, Paulina H. Wanrooij, Sjoerd Wanrooij, Nils-Göran Larsson, L. Marcus Wilhelmsson, Maria Falkenberg, and Claes M. Gustafsson. (2012). Mammalian transcription factor A is a core component of the mitochondrial transcription machinery.
Spelbrink JN, Toivonen JM, Hakkaart GA, Kurkela JM, Cooper HM, Lehtinen SK, Lecrenier N, Back JW, Speijer D, Foury F, Jacobs HT. (2000). In vivo functional analysis of the human mitochondrial DNA polymerase POLG expressed in cultured human cells. J Biol Chem. Aug 11;275(32):24818-28.

Sergerie m , G. Laforest, L. Bujan, F. Bissonnette, G. Bleau, .(2005). Sperm DNA fragmentation: threshold value in male fertility, *Human Reproduction*, Volume 20, Issue 12, 1 Dec, Pages 3446–3451,
Sharlip ID, Jarow JP, Belker AM, Lipshultz LI, Sigman M, Thomas AJ, Schlegel PN, Howards SS, Nehra A, Damewood MD, Overstreet JW, Sadovsky R. (2002). Best practice policies for male infertility. Fertil Steril. May;77(5):873-82.
Scarpulla RC. (2008). Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol Rev. Apr;88(2):611-38.
Said TM, Agarwal A, Sharma RK, Thomas AJ, Sikka SC. (2005). Impact of sperm morphology on DNA damage caused by oxidative stress induced by β-nicotinamide adenine dinucleotide phosphate. Fertil Steril. 83(1):95–103.
Sassone-Corsi, P.(1997). Transcriptional checkpoints determining the fate of male germ cells. Cell 88, 163–166.
Seidel-Rogol BL and Shadel GS (2002) Modulation of mitochondrial transcription in response to mtDNA depletion and repletion in HeLa cells. Nucleic Acids Res 30,1929–1934.
Song GJ, Lewis V. (2008). Mitochondrial DNA integrity and copy number in sperm from infertile men. Fertil Steril. Dec;90(6):2238-44.
Sousa AP, Amaral A, Baptista M, Tavares R, Caballero Campo P, Caballero Peregrín P, Freitas A, Paiva A, Almeida-Santos T & Ramalho-Santos J. (2011). Not all sperm are equal: functional mitochondria characterize a subpopulation of human sperm with better fertilization potential. *PLoS One* 6 e18112.
Sutovsky, P., and G. Schatten. (2000). Paternal contributions to the mammalian zygote: Fertilization after sperm-egg fusion. Int Rev Cytol 195: 1-65.
Thangaraj, K., Joshi, M. B., Reddy, A. G., Rasalkar, A. A., & Singh, L. (2003). Sperm mitochondrial mutations as a cause of low sperm motility. *Journal of Andrology*, *24*(3), 388-392.
Tsukihara, T. et al. (1995). Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 a. Science 269: 1069-1074.
Taverne, D. Merkus, A.J. Bogers, B. Halliwell, D.J. Duncker, T.W. Lyons(2018). Reactive oxygen species: radical factors in the evolution of animal life a molecular timescale from earth's earliest history to the rise of complex life, Bioessays 40 (3)
Ursini F, Maiorino M, Brigelius-Flohé R, Aumann KD, Roveri A, Schomburg D, Flohé L. (1995). Diversity of glutathione peroxidases. Methods Enzymol. 252:38-53.
Ursini F., Heim S., Kiess M., Maiorino M., Roveri A., Wissing J., Flohé L. (1999). Dual function of the selenoprotein PHGPx during sperm maturation. *Science* 285, 1393–1396
Valenti, D.; Vacca, R.; Moro, L.; Atlante, A. (2021). Mitochondria Can Cross Cell Boundaries: An Overview of the Biological Relevance, Pathophysiological Implications and Therapeutic Perspectives of Intercellular Mitochondrial Transfer. Int. J. Mol. Sci., 22, 8312.
Venkatesh S, Deecaraman M, Kumar R, Shamsi MB, Dada R. (2009). Role of reactive oxygen species in the pathogenesis of mitochondrial DNA (mtDNA) mutations in male infertility. Indian J Med Res. Feb;129(2):127-37.
Vertika S, Singh KK & Rajender S (2020). Mitochondria, spermatogenesis, and male infertility an update. *Mitochondrion* 54 26–40.
Wallace DC. (1992). Diseases of the mitochondrial DNA. Annu Rev Biochem; 61:1175–212.
Weinberg JB, Doty E, Bonaventura J, Haney AF. (1995). Nitric oxide inhibition of human sperm motility. Fertil Steril; 64:408–13.
WHO. (2010). World Health Organization laboratory manual for the examination and processing of human semen Fifth edition. WHO Press; 287.
World Health Organisation. Estimated Overweight and Obesity Prevalence, Males, Aged 15+. Geneva: World Health Organisation; 2010a.
World Health Organization (WHO). International Classification of Diseases, 11th Revision (ICD-11) Geneva: WHO 2018).
Wu H, Huffman AM, Whitcomb BW, Josyula S, Labrie S, Tougias E, Rahil T, Sites CK & Pilsner JR (2019). Sperm mitochondrial DNA measures and semen parameters among men undergoing fertility treatment. *Reproductive Biomedicine Online* 38 66–75.
Wu S. H., Oldfield J. E., Whanger P. D., Weswig P. H. (1973). Effect of selenium, vitamin E, and antioxidants on testicular function in rats. *Biol. Reprod*. 8, 625–629
Zhao Y, Li Q, Yao C, Wang Z, Zhou Y, Wang Y, Liu L, Wang Y, Wang L and Qiao Z (2006). Characterization and quantification of mRNA transcripts in ejaculated spermatozoa of fertile men by serial analysis of gene expression. Hum Reprod 21,1583–1590.
Yakes FM, Van Houten B. (1997). Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proc Natl Acad Sci U S A. Jan 21;94(2):514-9

Wang, K.Z., Zhu, J., Dagda, R.K., Uechi, G., Cherra III, S.J., Gusdon, A.M., et al., (2014). ERK mediated phosphorylation of TFAM downregulates mitochondrial transcription: implications for Parkinson's disease. Mitochondrion 17, 132–140.