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"CHARACTERIZATION AND QUANTIFICATION OF SEMEN RHEOLOGICAL PROPERTIES AND SPERM MOTILITY."

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Abstract

Infertility affects 15% of couples of reproductive age worldwide, with increasing prevalence rates that are, in many areas, such as USA and Europe, below the rate required to sustain the current population levels. In particular, a decrease of male fertility as well as semen quality has been registered in the last years.

Currently the spermiogram, the routine analysis of the seminal fluid, represents the backbone of semen characterization. However, the spermiogram is not sufficient to test the fertilizing capacity as it tends to just consider semen parameters such as concentration, motility and morphology of spermatozoa, neglecting other fundamental external factors and causes. In fact, it may happen that a seminal fluid classified as normospermic is not able to generate a pregnancy in cases where female problem can be ruled out. In such situations, we talk about male idiopathic infertility. The latter can be attributed to multiple damages to the semen, such as defects in spermatogenesis, hormonal problems, oxidative stress, and genetic abnormalities. The causes of these complications can be also highly affected by lifestyle. One possible way to increase the predictive power of the spermiogram, thus allowing to capture semen defects which go undetected in the current routine testing, is to improve the quantitative analysis of the semen rheological response by looking for a signature of pathological conditions.

In this work, a thorough analysis of semen viscoelasticity by well-established rheological techniques, as opposed to the operator-dependent methods used in routine testing, is carried out on samples which have been classified as normal or pathological according to their spermiogram. The results of this work suggest that elasticity, in addition to viscosity, plays a fundamental role in sperm motility and can be used as a marker of sperm transport capacity, thus inspiring advances in diagnostic methods and medical treatments for male infertility.

1. Introduction

Fertility refers to the reproductive capacity of living organisms by sexual reproduction, that is based on the unification of male and female gametes. In mammals, this requires that at least one sperm cell, among the hundreds of millions that flow towards the egg driven by biochemical signals, reaches the site of fertilization. Before standing a chance to fertilise an egg cell, there is a long and difficult journey for sperms, that should travel through the fallopian tubes, where they find a complex windy path and a likely hostile environment, due to the presence of immune cells and a complex fluid as suspending medium. And that is not all. The few sperms able to overcome these first obstacles should be subjected to morphological and biochemical changes and, as the last step, should interact with the egg in the oviduct [1, 2]. Thus, the success of the reproduction is based on a fragile balance between biochemical and physical processes that, in turn, can be affected by genetic and lifestyle factors. When the balance is not reached or is broken, we refer to infertility. According to the definition of the World Health Organization, infertility is "the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse", and many other definitions have been used in clinical practice as well as in demographic research [3]. The heterogeneity of the criteria used to define infertility results in the difficulty of estimating the prevalence of infertility worldwide. Moreover, the discrepancy between data from large-scale population surveys on infertility and the ones from epidemiological studies as well as the fact that infertility can be vaguely referred to *women*, *men*, *couples* or *individuals* makes the determination of the number of people affected by this problem even more complicated [4]. Despite this, a recent study based on findings of the global burden of disease (GBD) have assessed the burden of infertility in 195 countries and territories from 1990 to 2017, reporting that infertility affects 15% of couples of reproductive age worldwide [5-7], with increasing prevalence rates that are, in many areas, such as USA and Europe, bring fertility below the rate required to sustain the current population levels (see maps in *Figure 1*). More in details, almost 50% of infertility cases are only due to female factors, 30% are related to male factors and 20% to the combination of both male and female factors [7, 8]. In particular, a decrease of male infertility as well as semen quality, in terms of sperm concentration and motility, has been registered in the last years. However, the percentage referred to males could be underestimated, since in many

countries, due to cultural issue and patriarchal societies, infertility is not registered as a male-related problem, hindering the collection of reliable data [7].

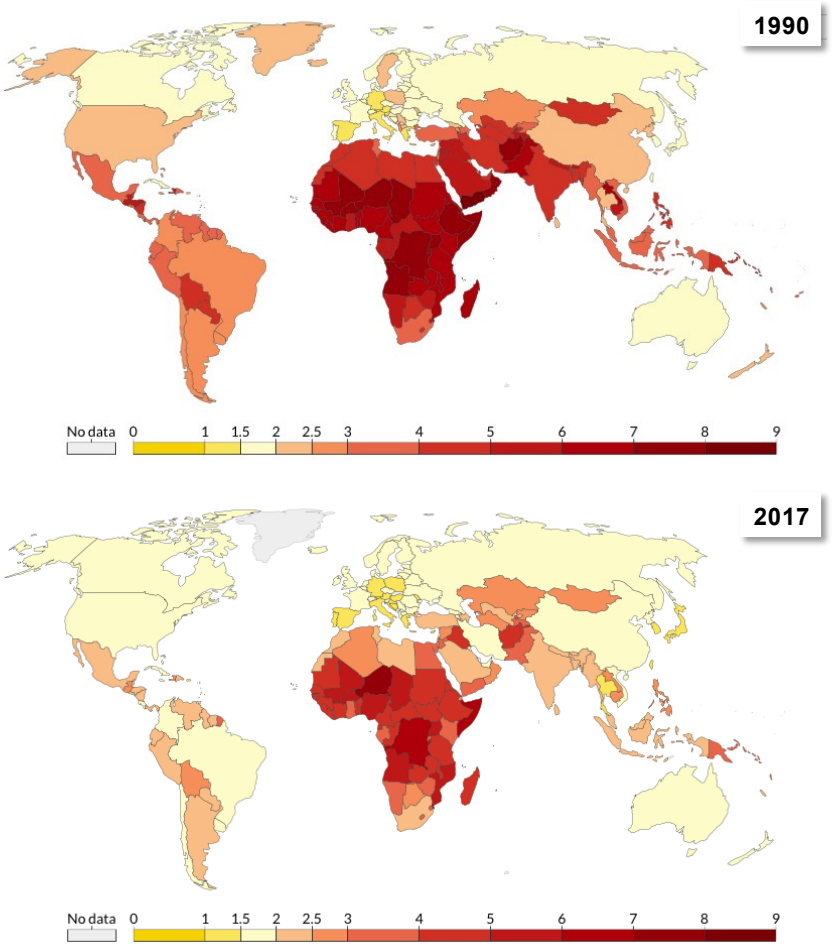

Figure 1. Total fertility rate, measured as the number of children that would be born to a woman, in accordance with age-specific fertility rates of the specified year, for 1990 (top) and 2017 (bottom) [9].

1.1. Seminal fluid and its characterization

Seminal fluid, also known as *sperm* or *semen*, is composed of spermatozoa, i.e. male reproductive cells, suspended in a liquid medium called *seminal plasma*. Spermatozoa are active swimming cells with a head of about 5 µm and a flagellum of approximately 55 µm [2] and represent 2-5% of the overall volume of seminal fluid, that correspond to 15-259 millions per milliliter. Spermatozoa synthesis takes place within the seminiferous tubules of the testes via spermatogenesis, a tricky process that can produce malfunctioning or unripe spermatozoa if even only one step of it fails. Once formed, spermatozoa are kept viable thanks to the help of seminal plasma, a complex fluid composed by liquids secreted

by the seminal vesicles (60-70%), prostate (20-30%) and bulb-urethral and urethral glands (<1%) [10].

Seminal plasma is essential for the maturation, metabolism and life of spermatozoa, as well as for their survival after ejaculation, guaranteed by the presence of molecules of different size (e.g. proteins[11], enzymes[12], lipids[13], prostaglandins[14], hormones[15], fructose[16], citric acid[17], vitamin C[18] and ions[19]). Each of them has a specific function. For example, fructose provides the necessary energy for normal sperm functions, while proteins are involved in several key steps preceding fertilizations, such as capacitation (i.e. the maturation process, involving molecular and cellular changes, that spermatozoa must undergo to acquire fertilization potential[20]), regulation, oviductal sperm reservoir establishment and sperm transport in the female genital tract. Another essential role of seminal plasma proteins is the modulation of the uterine immunological responses, both innate and adaptative[11]. Important roles are also played by zinc, which has antioxidative function and also acts as a bactericide and stabilizes the chromatin of spermatozoa [21, 22], by enzymes and citric acid, which intervene in the coagulation-liquefaction process of the sperm, and by bicarbonate, which modulates sperm motility and neutralizes the acidity of the vaginal environment [23]. In addition to sperm and seminal plasma, the ejaculate comprises also immature cells from spermiogenesis and flaking epithelial cells.

Despite the complexity, from both the chemical and physical point of view of such a fluid, the routine semen analysis, in addition to male detailed medical history and physical examination, focuses on the evaluation of just a few parameters [24, 25], as shown in Table I. The resulting report, called *spermiogram*, contains information from two types of tests: macroscopic and microscopic.

Macroscopic examination starts soon after semen liquefaction (i.e. the gel-like fluid formed by proteins from the seminal vesicles is broken up and the semen becomes thinner, homogeneous and watery) and allows the evaluation of semen volume, pH, viscosity. The precise measurement of semen volume is fundamental for the right calculation of sperm concentration. pH measurements are important since pH could affect sperm capacitation and motility [26]. Abnormal values of viscosity (i.e. the resistance of a fluid to deformation at a given shear rate [27]) could affect motility as well; in fact, high semen viscosity can

hamper sperm motility and could also lead to the incorrect determination of some parameters (i.e. sperm concentration, biochemical biomarkers) during routine tests. In the latter, semen viscosity measurement is carried out by only qualitative, operator dependent and not reproducible methods, consisting in the aspiration of the semen into a pipette, as shown in *Figure 2* and allowing the sample to drop by gravity. The length of the thread is used to classify viscosity as low, normal, and high .

Microscopic examination is carried out by using a phase-contrast microscope with a high-magnification objective, and is focused on the measurement of sperm number, motility, morphology and agglutination. *Sperm motility* is categorized in i) progressive -ideal-motility (P): active movement of spermatozoa, either linear or in large circles, irrespective of speed; ii) non-progressive motility (NP): absence of progressive movement of spermatozoa and iii) immotility: absence of movement (*Figure 3*).

Table I. WHO reference values for human semen [28].

Type of examination	**Parameter** (unit)	**Lower reference limit** (range)
Macroscopic	Semen volume (mL)	1.5 (1.4 – 1.7)
	pH	> 7.2
	Viscosity (semen wire length in cm)	< 2
	Time of liquefaction after ejaculation (min)	30 (15 - 60)
Microscopic	Total sperm number (10^6 per ejaculate)	39 (33 - 46)
	Sperm concentration (10^6 per mL)	15 (12 – 16)
	Progressive motility (PR %)	32 (31- 34)
	Total motility (PR+NP %)	40 (38 – 42)
	Vitality (live spermatozoa %)	58 (55 – 63)
	Sperm morphology (normal form %)	4 (3 – 4)
	Agglutination (spermatozoa per cluster)	< 10

PR=progressive; NP=non progressive.

Figure 2. Picture reporting the viscosity measurement in routine tests. The length of semen thread is used as an indirect measure of sample viscosity.

Normal *morphology* can be more difficult to quantify, due to the large number of shapes that spermatozoa can exhibit. Hence, potential fertilizing spermatozoa are taken as normal if they appear similar in shape (both head and tail curvature are taken into account) to those reported in the literature, that were recovered from endocervical mucus after intercourse. Regarding *agglutination*, it refers to the sticking of motile spermatozoa on each other, that could hinder the journey of sperm towards the egg [29].

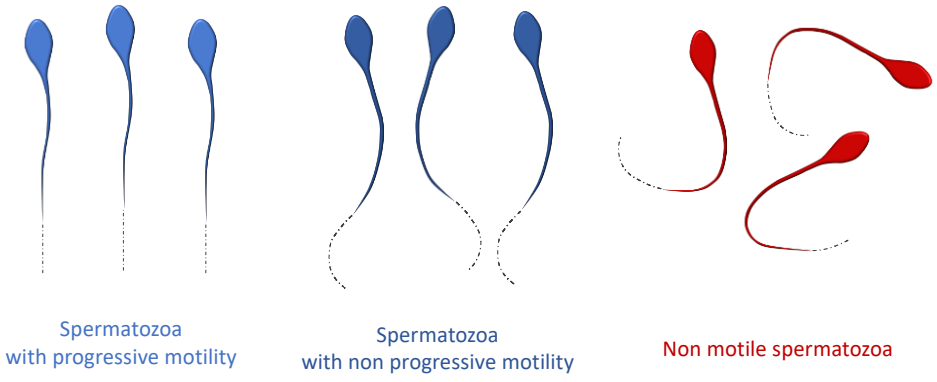

Figure 3. Sperm motility categorization.

1.2. Male infertility

In the big majority of male infertility cases (*Figure 4*), the cause can be found in the testes, the male reproductive glands that produce sperm and androgens (i.e. male sex hormones, primarily testosterone). Damage to the testes, resulting in altered sperm production, can be caused by many different conditions, including inflammatory states and infections, trauma, treatments for cancer (i.e. radiation/chemotherapy), and surgery. Abnormal sperm production can be also caused by genetic diseases (either inherited or acquired), which can lead to decreased mobility. Sperm production can also be affected by heat stress, environmental and lifestyle factors and, rarely, by hormone deficiency. Furthermore, about 30% of male infertility cases are of unknown cause and are then classified as *idiophatic* after spermiogram evaluation and routine medical examination. Indeed, it turned out that most of the idiopathic cases have roots in genetic alterations or in the combination of several factors. Several comprehensive reviews have been published so far related to the causes of male infertility [30] and the most recent ones have focused on genetic-related causes [31, 32].

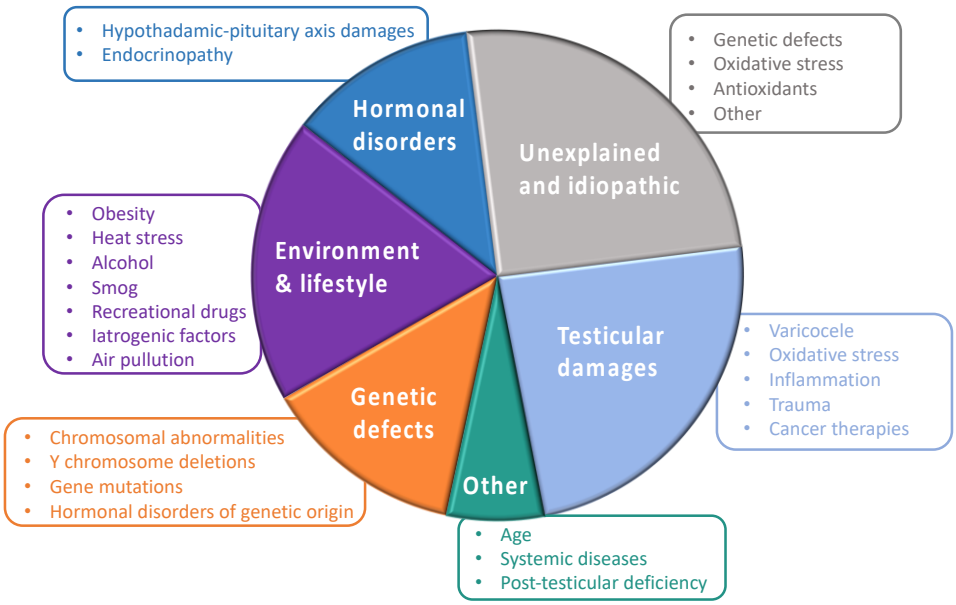

Figure 4. Most common male infertility causes.

1.2.1 Testicular damages

One of the most common testicular damages is due to **varicocele**, which is the pathological dilation of veins of the pampiniform plexus (i.e. a small veins network that ascends along the spermatic cord at the front of the ductus deferens, connecting an area of connective tissue at the back of the testes), which leads to reversed blood flow or impaired drainage of

the testicular vein that, in turn, results in raised testes temperature [33, 34]. It has been reported that varicocele exerts negative effects upon testicular function, as reflected by semen quality [30] [35], [36] and sperm function [37, 38], via various oxidative stress mechanisms [39]. Moreover, varicocele can cause chronic testicular, vas deferens (i.e. the tube that transports sperm to the urethra for ejaculation) or epididymal duct (i.e. coiled tube behind each testicle that transports sperm to the vas deferens) vasoconstriction and impaired spermatogenesis, sperm maturation and motility, due to the high concentration of catecholamine [40, 41]. Nevertheless, the negative effects of varicocele can be corrected by surgery (i.e. varicocelectomy) and most men affected by varicocele are able to reproduce, thus the exact connection between varicocele and infertility is still controversial.

Beyond being considered a central mechanism causing infertility in patients with varicocele, seminal oxidative stress is involved in many aspects of male infertility [42]**.** Indeed, while oxygen is fundamental for aerobic metabolism of spermatogenic cells, it can also be dangerous being at the basis of reactive oxygen species (ROS) production. Seminal oxidative stress is due to the imbalance between the production of (ROS) and antioxidant activity.

ROS overproduction can be due to intratesticular and post-testicular factors, like seminal leukocytes [43], residual cytoplasm or cytoplasmic droplet and abnormal spermatozoa, as well as to external factors, like alcohol, cigarette smoking, environment pollutants and several pathological conditions (e.g. varicocele and diabetes)[44].

Oxidative stress is one of the main causes of **DNA damages**, which can be of different nature (*Figure 5*), from nuclear and mitochondrial DNA defects to telomere attrition, epigenetic alterations (see section 1.2.4) and Y chromosomal microdeletions (see section 1.2.2).

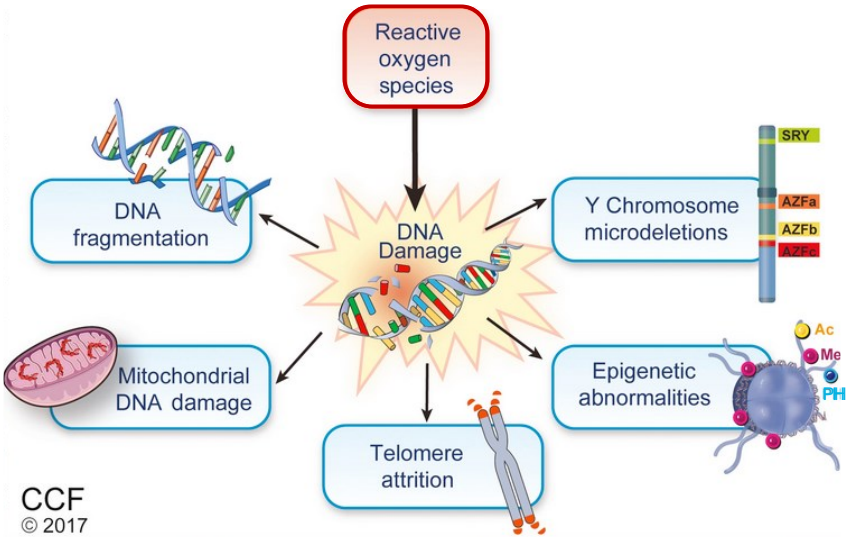

Figure 5. Types of sperm DNA damages. Adapted from[45].

DNA damages are due downregulation of DNA repair systems during late spermatogenesis. Indeed, oxidative stress can induce sperm chromatin/DNA damage, as demonstrated by recent studies that have correlated sperm DNA fragmentation and high levels of ROS in semen [46-48], while DNA integrity in germ cells is essential, since they have the task of passing the genome to the next generation [49]. In turn, DNA fragmentation can accelerate the process of germ cell apoptosis, resulting in low sperm count and in the deterioration of semen quality, compromising the potential of sperm to fertilize an oocyte and develop into a healthy embryo [50-52].

Mitochondria, which are largely present in middle piece of sperm cells with the task of supply energy for their motility, are also involved in ROS production and, in turn, high levels of ROS in semen can affect spermatozoa mitochondrial integrity [53], closing a vicious cycle. Indeed, spermatozoa with mitochondrial DNA (mtDNA) mutations have been found in case of high level of ROS [45-53].

Further targets of ROS are telomeres, due to their rich guanine structure and low oxidation potential. Telomeres are the cap-like structures present at the end of the chromosomes, and are essential for ensuring the integrity of normal genomic structure. Oxidative stress may induce the gradual loss of telomeres, known as telomere attrition, a process that limits the number of times cells can divide, slowly leading to the exhaustion of cells population in vital organs [54, 55].

Thus, it seems that there is a direct connection between pathological spermatozoa and ROS overproduction, which find a common origin in the sperm membrane remodeling process during spermatogenesis [56]. Furthermore, ROS overproduction has been identified as an etiological factor of idiopathic man infertility, as demonstrated of low antioxidant activity in sub-fertile men [57-59].

Inflammatory damages to male reproductive tract lead to ROS increased productions, that must be continuously inactivated by seminal plasma antioxidants. Indeed, inflammatory reactions to several factors, such as ejaculatory duct obstruction, epididymitis (usually caused by Chlamydia trachomatis and Escherichia coli infections [60]), urethritis, orchitis [61] (also due to mumps), chronic prostatitis, testicular torsion, varicocele, and drug therapy could result in reduced semen quality [62]. This is commonly due to the presence of pro-inflammatory cytokines, tumor necrosis factor (TNF) and interleukins, which have a key role in controlling immune cell function in physiological conditions, but could become dangerous when their level is higher than normal, as happens in inflammatory conditions [63]. However, cytokines produced by non-immune cells, such as testicular somatic and spermatogenic cells, both under normal conditions and in response to inflammatory stimuli, play also a fundamental role in the stimulation and maintenance of spermatogenesis (for comprehensive review see [64]), and any imbalance in their production may lead to infertility [63]. Testes inflammatory processes could also lead to leukocytospermia (i.e. high concentration of white blood cells in semen) that, in turn, is directly related to the concentration of immature germ cells and spermatozoa with normal morphology [65].

Further testicular damages can be induced by therapeutic treatments for **cancer therapy**, such as chemotherapy and radiation therapy [66]. Indeed, it has been established that chemotherapy drugs (e.g. cyclophosphamide [67]) can strongly affect spermatogenesis [68-70] by developing permanent gonadal damage [71, 72], while radiations can induce permanent injuries to germ cells. If radiations are used *in situ* (e.g. for the treatment of testis carcinoma), permanent sterility can likely occur [68, 69].

1.2.2 Genetic disorders background

Men reproductive process involves a complex coordination of a wide range of genes, and alterations in any of these genetic-based pathways can lead to infertility. Genetic disorders

are at the base of 10% - 30% cases of male infertility [73-76], and can compromise spermatogenesis (where at least 2,000 genes are involved in [32]) and sperm transportation to the egg for fertilization. The primary types of genetic disorders responsible of male infertility are related to chromosomal abnormalities (e.g. Klinefelter syndrome), Y chromosome deletions, mutation of cystic fibrosis gene and endocrine disorders of genetic origin.

Chromosomal abnormalities can be numerical and/or structural, can involve either sex chromosomes or autosome via their interference with meiosis chromosome synapsis or can be due to chromatid pairs during mitosis or to chromosomal lagging during anaphase [32, 77-81], and likely influence spermatogenesis. They are mainly found in men with severe oligozoospermia (i.e. low number of spermatozoa) and azoospermia (i.e. absence of spermatozoa), the risk progressively decreasing with increasing sperm output. The most frequent numerical chromosomal abnormalities of the karyotype is Klinefelter syndrome, which is characterized by the presence of one or more extra sex-determining X chromosome [82, 83] (*Figure 6a*) and its variants, such as mixed gonadal dysgenesis, which results from the sex-determining Y-chromosome mosaicism (i.e. some cells with a typical number of chromosomes and some with an incorrect number of chromosomes [67]) and leads to abnormal gonadal development [84]. Y chromosome is the smallest human chromosome and consists of short (p) and long (q) arms, which contains the gene SRY (Sex-determining Region Y) triggering male development (*Figure 6*). 46,XX male syndrome (SRY+) is due to the translocation of SRY gene from Y chromosome to the X chromosome during meiosis when the two sex chromosomes recombine (*Figure 6a*). Men with 46,XX male syndrome may present maldescended testes, gynaecomastia and ambiguous genitalia, and are azoospermic with no exceptions [32, 85].

Among structural chromosomal abnormalities, the most common on the autosomes is known as Robertsonian translocation, which is due to the fusion of two acrocentric chromosomes (usually chromosomes 13 and 14 or chromosomes 14 and 21), resulting in a single abnormal chromosome, generally dicentric, containing most of the long arms of the original two, and subsequent loss of their short arms (*Figure 6b*). Robertsonian translocation does not manifest phenotypically, even if it can affect fertility and/or the outcome of pregnancy due to possible incorrect gametogenesis [86].

Y chromosome microdeletions represent other common genetic causes of male severe infertility and are referred to deletions of azoospermia factor (AZF) regions (AZFa, AZFb, and AZFc) that are present on the q arm of Y chromosome (*Figure 6c*). Deletion of AZFa results in complete azoospermia, loss of AZFb leads to spermatogenesis interruption and deletion of AZFc results in azoospermia or oligospermia [86, 87]. It is worth mentioning that men with infertility caused by genetic factors, such as Y chromosome deletion, can transmit the problem to their sons, who would also have infertility [31, 88].

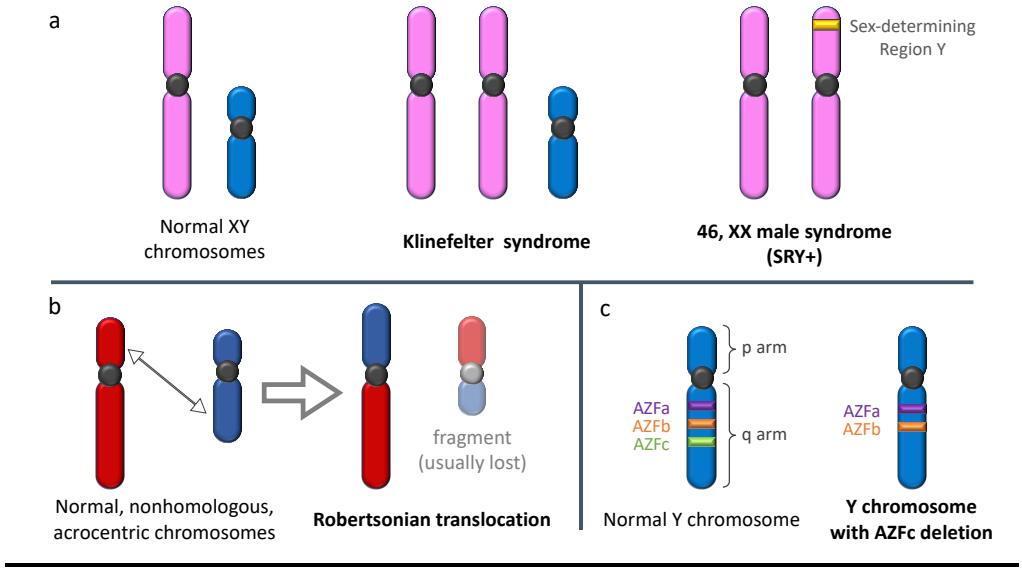

Figure 6. Schematics of some of the more common chromosomal alterations causing male infertility.

Gene mutations. Further factors at the base of infertility have been recognized in the mutations of certain genes, the most common being the CFTR (cystic fibrosis transmembrane conductance regulator) gene and the androgen receptor gene. Cystic Fibrosis (CF) is the most frequent life-limiting genetic disease in Caucasian populations, and it is caused by mutations in a gene that encodes CFTR protein regulating the exchange of Cl^- and Na^+ ions across epithelial membranes [89]. Patients suffering of CF show severe respiratory problems, but CF also affects intestine and exocrine glands, leading to multi-organ failure as well as to infertility. Males carrying CFTR mutations can have congenital bilateral absence of vas deferens (CBAVD)[90] or poor sperm quality, that lead to azoospermia, oligospermia and teratozoospermia (i.e. high percentage of spermatozoa with abnormal morphology).

Moreover, men suffering from infertility of unknown cause may carry relevant mutations in the androgen receptor (AR) gene and in the estrogen receptor genes (ESR1 and ESR2). The former (AR) is essential for development and maintenance of the male phenotype and spermatogenesis [86]. AR is located on the X chromosome and can lead to a variety of defects, collectively known as androgen insensitivity syndrome, indicating a variety of defects that range from un-equivocally female, without any androgenic effects, to partial androgen insensitivity characterized by genital ambiguity, up to males with under virilization [86, 91]. The ESR1 and ESR2 genes are involved in the physiological responses to estrogens that, in males, are synthesized from testosterone. Although estrogens are usually seen as female-related hormones, they can strongly affects male reproductive systems; indeed, estrogens concentration in semen is higher than that in serum of women [92]. Thus, findings on the correlations between estrogen insufficiency and abnormal spermatogenesis have led to the investigations of the role of ESR1 and ESR2 in male infertility. However, results are still controversial [31].

Hormonal disorders of genetic origin. Low levels of testosterone in the serum could be an index of hypogonadism. When it is due to genetic disorders (e.g. Klinefelter's syndrome) or testicular damages (e.g. trauma, orchitis, cancer therapies) is called hypergonadotropic hypogonadism or primary hypogonadism (i.e. the problem comes from the gonads), while when it is due to a congenital defect in the hypothalamic-pituitary-gonadal axis it is known as congenital hypogonadotropic hypogonadism (CHH). CHH is a rare (incidence of 1 in 8,000 men) genetic disease with variable expressivity, penetrance, and inheritance. It may results in delayed puberty and reproductive disorders due to the deficiency in hypothalamus secretion of the gonadotrophin-releasing hormone (GnRH) [32], which, in turn, is involved in follicle-stimulating hormone (FSH) and luteinizing hormone (LH) release from pituitary gland [93]. From the genetic point of view, CHH presents a number of challenging features for genetic analysis, since some genes involved in it (e.g. FGFR1 and PROKR2) might cause two different phenotypes (i.e. Kallmann syndrome [93, 94] and normospermic CHH [95]) and also because CHH does not always follow the Mendelian inheritance rules, as it can be caused by digenic or oligogenic transmission. Finally, it has been found that CHH can be reversible by suspending testosterone therapy [96]. Genetic

variants (i.e. polymorphism or deletion) in the sex hormone-binding globulin (SHBG) gene and in the follicle stimulating hormone (FSH) receptor (FSHR) gene have also been studied for a possible role in spermatogenesis. Indeed, SHBG gene is involved in both delivering sex hormones to target tissue and controlling the concentration of androgens in the testes [93] and FSHR codes for the receptor of FSH, an hormone essential for the normal functionality of gonads [75, 97].

In summary, it is clear that a big number of cases of male infertility, taking also into account idiopathic cases, may have complex genetic origins. Thus, in depth investigation of the underlying genetic defects responsible for male infertility should be considered, exploiting advanced diagnostic technologies, such as next-generation sequencing (NGS) [76, 98] and preimplantation genetic diagnosis (PGD) [99, 100].

1.2.3 Hormonal imbalance

Rarely, infertility can be found its origin in defects of the central hormonal regulation of testis function, that lead to hormonal imbalance or deficiency. The defects can be genetic (as seen in the previous section) or acquired (e.g. due to tumors, infiltrative diseases, empty sella, and iatrogenic and autoimmune hypophysis) [32]. Indeed, when hypogonadism is caused by gonadotropin deficiency or dysfunction as a result of disease or damage to the hypothalamic-pituitary-gonadal axis (*Figure 7*) is called hypogonadotropic hypogonadism, central hypogonadism, or secondary hypogonadism, since the problem lies in the brain rather than in gonads, in particular in hypothalamus and pituitary gland (hypophysis) [101]. The latter has the key function, among other, of synthetizing and secreting hormones such as LH, FSH that, in turn, drive the production of testosterone and sperm from testes. Any condition that tends to lower LH and FSH levels, such as a pituitary tumor, can result in spermiogenesis defects and low testosterone levels in the serum [101].

Another indicator of abnormal spermatogenesis can be the testosterone/estradiol ratio. Indeed, decreased serum testosterone/estrogen ratio, together with decreased testosterone and increased estradiol than fertile controls, has been found as peculiar of endocrinopathy in subjects with impaired sperm production[93].

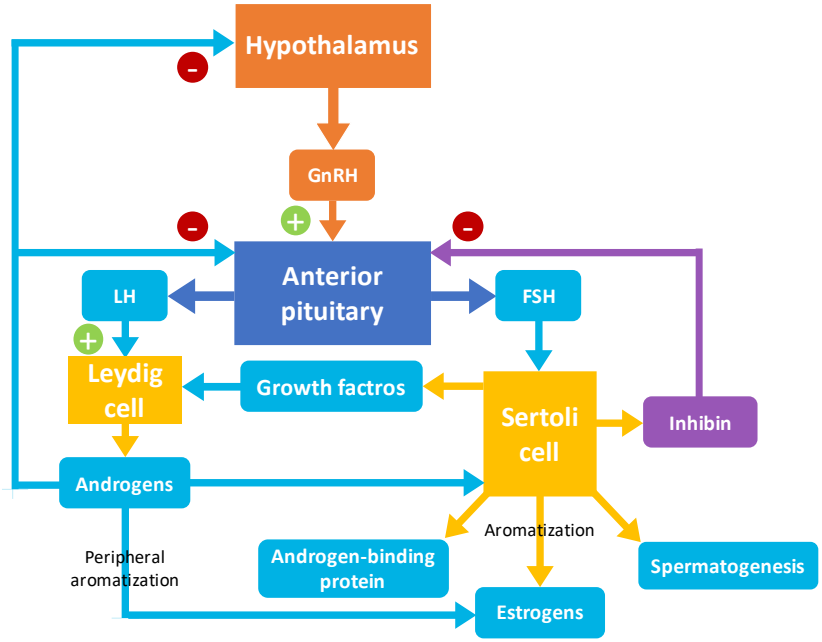

Figure 7. Hypothalamic-pituitary-gonadal axis and its functions.

1.2.4 Environmental and lifestyle factors

Detrimental lifestyle practices and pollutant exposure registered in the last decades have been recognized as key culprits for the reduced quality of semen at the base of man infertility all around the world. Factors like obesity, heat stress, alcohol and smoke abuse, air pollution are just few of the causes that may result in dramatic impairment of spermatogenesis.

Obesity. One of the most alarming problem leading to man subfertility in modernized societies is obesity, overweight men (i.e. with high body mass index - BMI>30 Kg/m2) [102] showing increased likelihood of low testosterone level, impaired spermatogenesis, sperm DNA damages and reduced success of conception [103, 104]. Different mechanisms can be associated with the effects of obesity on male infertility, such as hormonal imbalance, sleep apnea, and increased scrotal temperature [105]. It has been found that there is an inverse correlation between BMI and the fertility-related hormones, since in obese men serum high levels of estrogen and low levels of FSH, LH, inhibin B and free and total testosterone [106], typical of hypogonadotropism, are present. In obesity, hyperestrogenemia can be in part ascribed to the increased mass of white adipose tissue, which is responsible for the activity of aromatase, a key enzyme involved in the conversion of testosterone into estradiol. Thus, the higher the amount of adipose tissue, the higher the concentration of estradiol that might have a part in dysregulation of the hypothalamic-pituitary-gonadal axis (*Figure 7*). In

particular, high levels of estrogens negatively affect hypothalamus by altering GnRH pulses, responsible of the release of FSH and LH from pituitary gland that, in turn, have an impact on the release of testosterone from gonads [106, 107]. Another adipose-tissue specific factor is resistin, that induces insulin resistance [108]. The main function of insulin is metabolic, promoting the entry of glucose into cells. In the brain, insulin is responsible of food intake regulation, reducing the feeling of hunger (similarly to leptin)[108]. This mechanism does not work in obesity, where insulin resistance (as in the case of type 2 diabetes) occurs, meaning that the cells in the body decrease their sensitivity to the action of this hormone, resulting in an overproduction of insulin by the pancreas. When the insulin produced is so high to overcome cellular resistance, all the glucose quickly enters the cells and accumulates in the form of fat, further increasing adipose tissues and raising estrogens level [109]. High insulin level hinders the synthesis of SHBG, inhibiting the biologic activity of testosterone and estradiol.

Impaired pituitary gonadal function has been also associated with sleep apnea, a sleep disorder characterized by pauses in breathing during sleep, common in obese men. Sleep apnea could lead to upper airway obstructions and hypoxia that, in turn, causes the disruption of nocturnal testosterone rhythm and decreasing morning testosterone levels compared to controls [108, 110].

Heat stress. Heat can affect sperm production as well. Indeed, normal testicular function depends on temperature, that should be lower than that of body thanks to the fact that testes are located in the scrotum (i.e. outside the body)[111]. The increase of testicular temperature can be caused by clinical disorders, such as cryptorchidism (i.e. failed descent of testes from their location in abdominal cavity before birth)[112] and varicocele, occupational heat exposure (i.e. bakers and welders, professional drivers)[112] and lifestyle (e.g. exposure of genitals to radiation through cell phones and laptops, tight-fitting underwear, hot baths)[113] and can lead to poor semen quality and sperm DNA damages [114].

Alcohol. It has been found that chronic and heavy (> 20-25 units per week) alcohol consumption has a negative effect on spermatogenesis and consequently on semen quality, oligozoospermia, asthenozoospermia and teratozoospemia being often found in chronic male alcohol consumers [48, 113]. This is likely due to the inhibiting effect of ethanol on LH release, which results in a significant reduction of testosterone that, in turn, leads to

estradiol increase and to impaired semen quality. However, it is still not clear at what level of the hypothalamic-pituitary-gonadal axis ethanol exerts its suppressive function [115].

Smoke. Another bad habit considered as a male fertility risk factor is smoking, acting via different mechanisms. Indeed, tobacco combustion produces deadly toxic chemical compounds that could affect germ cells, leading to asthenozoospermia (i.e. reduced sperm motility) and to sperm apoptosis [116]. Moreover, smoking can reduce spermatozoa mitochondrial activity, hampering fertilization capacity. The increase of seminal leukocytes, which are the major source of ROS in the ejaculate, has also been associated with cigarette smoking, resulting in chromatin and/or DNA damages [117, 118]. Finally, possible degeneration of Leydig cells has been reported after prolonged smoking, leading to reduced production of testosterone [118, 119]. However, the effect of cigarette smoke on testosterone levels is still under debate.

Recreational drugs. The use of recreational drugs, such as cannabis, cocaine, opioids narcotics, anabolic steroids, may have the adverse effect of impairing the functionality of the hypothalamic-pituitary-gonadal axis, inducing hypogonadism, defects in spermatogenesis and reduced sperm motility [113, 120].

Iatrogenic factors. Beyond drugs used for chemotherapy, there are a number of drugs of common use and surgical treatments that may cause temporary male infertility, which in this case is known as iatrogenic infertility. Drugs can impair male fertility acting through diverse mechanisms, such as direct effect on germ cells, unbalancing the delicate equilibrium of the hypothalamic-pituitary-gonadal axis, damaging erectile or ejaculatory function, and decreasing the libido [67, 121].

Just to make some examples, sulfasalazine (used for inflammatory bowel disorders), impairs sperm quality in terms of spermatozoa concentration, motility and morphology [122]; cimetidine (used for dyspepsia) interferes with sperm production [123]; sex steroids (i.e. estrogens and testosterone) and GnRH analogs reduce gonadotropin secretion, leading to azoospermia [124, 125]. However, the action of these drugs is usually reversible, semen parameters returning normal upon cessation of the therapy.

Air pollutants. Exposure to air pollutants is an additional factor that must be included among possible causes of male reproductive problems. Some common environmental pollutants such as bisphenol A (BPA), phthalates, dioxins, polycyclic aromatic

hydrocarbons (PAHs), toxic metals (e.g. Cu, Pb, Zn) and pesticides are included among substances that exert endocrine disruptors (EDs) activity[113]. Indeed, EDs can affect both the hypothalamic-pituitary axis and testicular spermatogenesis [126] (e.g. diesel exhaust particles contain EDs that can affect gonadal steroidogenesis and gametogenesis [127]), resulting in poor sperm quality. Other substances, such as NO_2, O_3 and particulate matter can generate ROS that, in turn, may cause DNA damages. Indeed, an association between air pollution and increased DNA damage has been found, even in absence of alterations of semen quality [128, 129]. Finally, there is some evidence suggesting that air pollutants could trigger epigenetic modifications, which are heritable changes in gene expression and in phenotype that do not involve alterations in DNA sequence. Epigenetic changes include DNA methylation (i.e. addition of methyl groups to DNA cytosines [130, 131]), histone (i.e. basic protein that help condense DNA into chromatin) modifications (i.e. covalent post-translational modifications to histone proteins [132]) and post- transcriptional regulation by noncoding microRNAs [133]. Although the direct effect of environmental pollutant exposure on epigenetic state has not yet proved, the data available on animals or on organs and tissues in humans (such as bronchial epithelial cells [134] and blood [135]) strongly suggest that air pollutants could negatively affect the epigenetic mechanisms that are involved in spermatogenesis, leading to both poor semen quality and, in turn, to transgenerational defects (i.e. alterations of embryo development and congenital diseases)[136].

1.2.5 Idiopathic infertility

Up to 40% of male infertility cases are of unknown aetiology. In those cases, we talk of idiopathic male infertility, which is characterized by normal physical examination and endocrine laboratory testing and no evident history of fertility problems, but sperm abnormalities from semen analysis of idiopathic causes by established standard testing protocols. The term unexplained infertility, instead, refers to those cases in which spermiogram values are normal and a female infertility factor could not be identified [137]. It has been suggested that idiopathic male infertility (IMI) associated to ROS results from factors at both genetic and proteomic level, which are not evaluated in infertility routine analysis. As shown in *Figure 8*, genes associated with IMI have been grouped in four sets,

namely genes associated with IMI without association with single-nucleotide polymorphisms (SNP); genes associated with IMI with association with SNPs [138]; ROS genes (the big majority)[42, 139] and antioxidant (AO) genes [140].

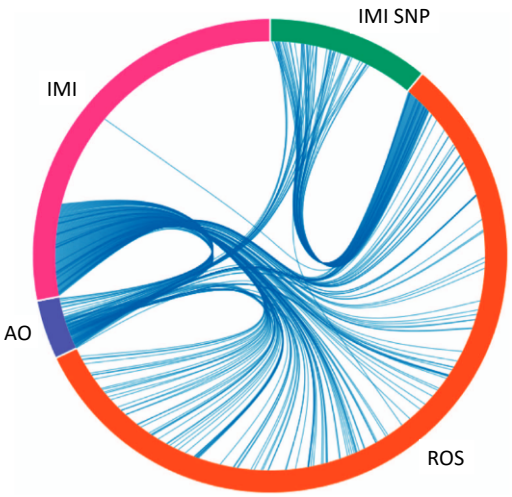

Figure 8. Shared or common genes among four gene sets associated with idiopathic male infertility (IMI). IMI: 484 genes associated with IMI with no single-nucleotide polymorphisms (SNP) associated with it; SNP: 192 IMI genes with SNPs; ROS: 981 ROS genes; AO: 70 antioxidant genes. Adapted from[137].

The genes that are part of these sets encode for proteins that could serve as markers useful for the diagnostic and therapy of IMI [137]. The negative effect of ROS usually can be found among DNA damages that result in impaired motility and abnormality of sperm morphology while AO have the positive protective function against ROS damaging effects. However, as said before, ROS and AO imbalance leads to DNA damages. The distribution of SNP across genes also plays a key role in IMI, most of all resulting in impaired spermatogenesis and sperm motility.

1.2.6 Other

Additional factors affecting male fertility can traced back to oxidative stress and testicular and post-testicular damages. Indeed, it has been shown that aged men may present higher DNA fragmentation [141] than younger subjects, likely due to oxidative stress [142] and testicular apoptosis of germ cells, that leads to the decline of both potential daily sperm production and Leydig cell function [143]. However, other sperm parameters, such as concentration, motility and morphology do not change significantly with men age. Systemic

diseases, such as chronic renal and liver failure, may impair men fertility. In particular, renal failure may affect either hypothalamic–pituitary–gonadal axis or testes, leading to both endocrine and exocrine testicular dysfunction [144, 145], while cirrhosis can entail testicular atrophy and sexual dysfunctions [146]. In both cases, transplantation of kidney and liver can be resolutive.

Regarding post-testicular defects, acquired or congenital obstruction arising from the epididymis, vas deferens, or ejaculatory duct can be at the basis of post-testicular deficiency, also known as obstructive azoospermia, present in about 40% of men with azoospermia [147]. In contrast with testicular damages, post-testicular abnormalities are treatable, and it is often possible to achieve the restoration of fertility potential.

1.3. Semen viscosity and viscoelasticity

After ejaculation, semen coagulates and, immediately afterwards, a gradual liquefaction starts (after about 20-30 minutes of ejaculation the semen is liquified) and semen viscosity decreases [148]. However, in some cases, semen viscosity stays high, and it is regarded as semen hyperviscosity that is considered as an index of impaired fertility. Indeed, it is thought that semen hyperviscosity can hinder normal sperm movement in the female reproductive tract. Some pathological conditions [149], either genetic, such as cystic fibrosis, or acquired, such as sex glands disfunction, oxidative stress, inflammation and infections and leukocytospermia, have been correlated to the presence of hyperviscous semen, all resulting in decreased semen quality, in particular reduced sperm motility [150], and impaired fertility rate [151] (*Figure 9*).

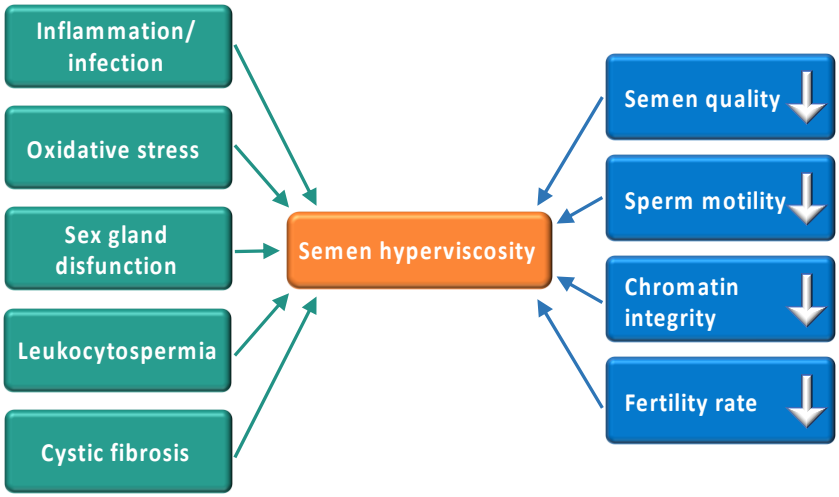

Figure 9. Causes and effects of semen hyperviscosity.

Concerning genetic causes (i.e. cystic fibrosis), CFTR mutation that causes CF pathology results in defects in the regulation of chloride and sodium ions exchange across epithelial membranes that, in turn, affect the fluidity of many body fluids, such as blood, amniotic fluid, synovial fluid and mucus [152]. Regarding other causes, in many cases a correlation has been found between semen hyperviscosity and disfunction of periprostatic venous plexus [153], prostate and seminal vesicles [154, 155].

Seeking the aetiology and evaluating the consequences of semen hyperviscous nature is made more difficult by the fact that, currently, the routine semen viscosity measurement consists in the aspiration of the semen into a pipette and allowing the sample to drop by gravity. The length of the thread is used to classify viscosity as low, normal, and high, considering normal viscosity when thread length is < 2 cm. It is worthy to note that such a procedure, more than a measure of viscosity, likely evaluates the combined effect of the elastic and viscous properties of the fluid. Despite the aforementioned issues, many studies focused on the correlation between semen hyperviscosity and the possible causes/effects of it by using semen thread length evaluation[148]. Several investigations associated leukocytospermia in infertile patients to semen hyperviscosity, patients with hyperviscous semen showing higher percentage of leukocytes compared with controls, and reduced sperm motility and vitality [154]. Other studies [156, 157] reported a correlation between semen hyperviscosity and severe impairment of low- and high- molecular weight seminal antioxidants (e.g. enzymatic reactive oxygen species -ROS- scavengers that have the function to protect spermatozoa against ROS toxicity). In turn, ROS overproduction can be

due to high levels of leukocytes present in infections or inflammations. Semen hyperviscosity has been also associated to dysfunction of the male accessory glands. In particular, it has been observed that quality semen, in terms of sperm motility, vitality, and fructose level were impaired in high viscous samples from donors with hypofunction of the seminal vesicles [155].

So far, only few papers reporting the use of quantitative methods have been proposed for the rheological characterization of human semen, mostly exploiting glass capillary viscometers [153]. Tjioe and Oentoeng [158] used a capillary viscometer to correlate semen viscosity and motility, finding a decrease of sperm motility with increasing viscosity. Ray et al. [159] studied the correlation between spermatozoa concentration and semen viscosity, observing the same viscosity for normal, azoospermic (absence of spermatozoa), and vasectomized patient's samples, and higher viscosity for oligospermic patients. A possible explanation of this result can be ascribed to the decrease of viscosity of semen due to the presence of swimming, pusher cells, as discussed in the following [160]. Dunn and Picologlou [161, 162] used a multiple-point capillary viscometer for the evaluation of the time-dependent viscoelastic behavior of semen, even if it was a only one-donor study. They showed that semen behaves as a viscoelastic fluid immediately after ejaculation and as a Newtonian fluid after full liquefaction. Aydemir et al. [163] studied the effect of malondialdehyde (MDA), an unsaturated carbonyl products of oxidative stress, on semen viscosity, reporting a significant positive relationship of seminal fluid viscosity with seminal plasma MDA and sperm MDA [157, 163] (*Figure 10*, top panel) and Siciliano et al. reported a correlation between semen hyperviscosity and severe impairment of low- and high-molecular weight seminal antioxidants (e.g. enzymatic ROS scavengers such as Cu/Zn superoxide dismutase (SOD), catalase (CAT) and chain-breaking antioxidants such as ascorbate, urate, albumin, glutathione, and taurine, which have the function to protect spermatozoa against ROS toxicity (*Figure 10*, bottom panel)[156].

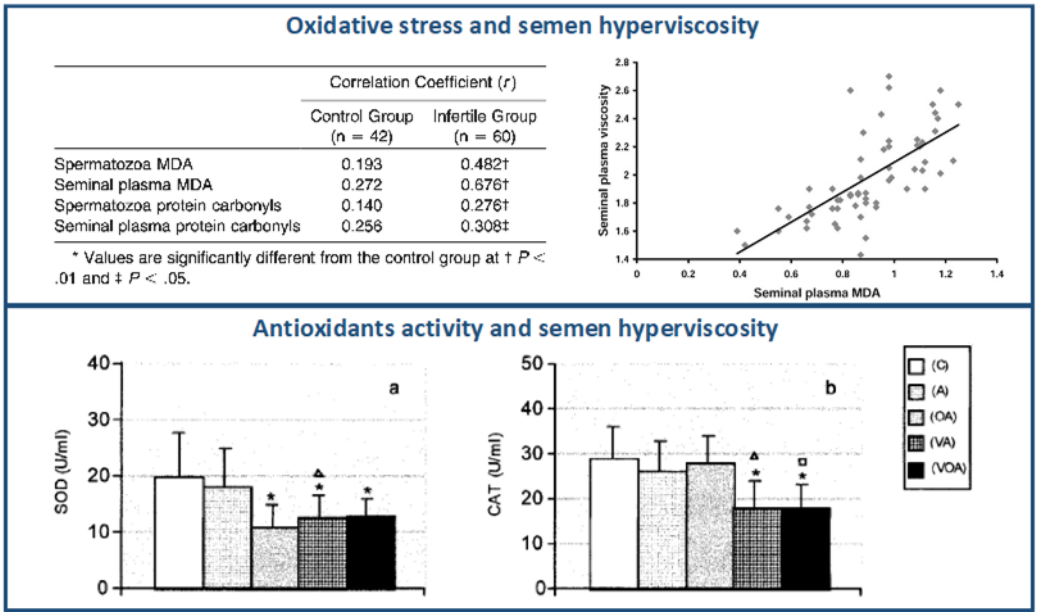

*Figure 10. Top panel, left: table reporting the relationship between semen viscosity and spermatozoa MDA, seminal plasma MDA, spermatozoa protein carbonyls, and seminal plasma protein carbonyls; top panel, right: graph reporting semen viscosity (mPas) as a function of and semen MDA levels (nmol/mL) for the 60 infertile males. Adapted from[163]. Bottom panel: Enzymatic antioxidant activity in seminal plasma of asthenozoospermia (A), oligoasthenozoospermia (OA), hyperviscous asthenozoospermia (VA), and hyperviscous oligoasthenozoospermia (VOA), for a. SOD and b. CAT. * indicate significant difference (P<0.1) as respect to control value; square symbol indicates significant difference (P<0.1) as respect to A; triangle symbols indicate significant difference (P<0.1) as respect to OA. Adapted from[156].*

However, also capillary-based approaches are not the right tools for the assessment of viscosity of a complex fluid as human semen is. In fact, it has been observed that semen viscosity is not constant with the imposed shear rate, but decreases, showing a behavior typical of viscoelastic fluids [164, 165]. Indeed, viscoelastic behavior is common in biological micro-structured fluids, such as blood [166] and mucus [152]. Thus, more reliable viscosity measurements have been performed by using rotational viscometers. Hubner et al.[165] used a Couette viscometer to measure the viscosity of previously frozen semen samples at 3 different shear rates, classifying the samples according to the spermatozoa concentration, and finding a non-Newtonian behavior for all the samples but no correlation with sperm count. Similar results have been reported in the work of Lin et al. [150], except for the higher viscosity found for oligospermic patients. More recently, Mendeluk et al.[164] reported a pseudoplastic behavior for semen, providing shear stress *vs* shear rate curves for both

normal and hyperviscous samples, ascribing hyperviscosity to the presence of a highly organized network of macromolecules.

Regarding the effect of semen abnormal physical properties, it has been found that altered semen viscosity could strongly affect spermatozoa progressive motility [167], as happen for many swimming microorganisms, such as bacteria and algae [168]. In fact, it has been observed that physical environmental factors have a role in shaping the evolution of spermatozoa flagellar kinematics. In particular, the increase of suspending medium viscosity decreases the wavelength and the frequency of spermatozoa flagellar wave [8, 169, 170], while larger uniform flagellar beating amplitude and localized regions of high curvature optimize swimming efficiency [171, 172]. In turn, fluid viscosity can be influenced by the presence of actively swimming micro-organisms as sperm cells. It has been found that the viscosity in elongational flow of suspensions of pullers (e.g. algae) is higher than that of a passive suspension (i.e. a suspension of inactive cells of the same size, shape and concentration), while viscosity of suspensions of pushers (i.e. spermatozoa of mice and bacteria) is lower than that of a passive suspension [160].

Furthermore, the elastic nature of semen must be considered: in fact, the forces exerted in a viscoelastic medium on a flexible swimmer are different from those exerted by a Newtonian one [173]. The elastic nature of semen could originate from interactions among spermatozoa as well as from the presence of macromolecules in plasma semen, as it happens for airways mucus [152]. As shown in Creppy et al. [174], even after strong dilution, both semen and plasma semen show the same shear-thinning trend that could likely be ascribed to the presence of proteins. This means that the motility of spermatozoa in such a complex medium could be altered in a way that is still not completely analyzed and treated, especially considering that increased viscoelasticity could be due to a plethora of causes such as inflammatory states and gland disfunctions.

Viscoelastic properties of the suspending medium play a key role in spermatozoa selection as well as in spermatozoa swimming efficiency in mucus in the female reproductive tract [175]. Some studies focused on the swimming of spermatozoa in highly viscoelastic media, mimicking the behavior of mucus of the female reproductive tract. By numerical modeling, it has been found that the viscoelastic nature of the medium increases spermatozoa velocity by forming regions of highly strained fluid behind the tail [176]. However, the used model,

although effective in capturing the elastic responses, was not able to capture the shear thinning behavior of the fluid nor the effects of macromolecules that induce viscoelasticity. In a recent study - on bull spermatozoa in a model viscoelastic fluid - it has been observed how the viscoelastic nature of the medium can increase the efficiency of spermatozoa swimming and can also induce dynamic clustering and collective swimming, likely contributing to effective fertilization [177] *Figure 11.* Furthermore, Creppy et al. [178] used livestock semen to analyze the swimming efficiency of concentrated suspensions of spermatozoa in confined conditions, mimicking the confined environment of the oviduct tract. They found a non-trivial spontaneous motion of spermatozoa, such as a phase transition at a critical volume fraction of cells.

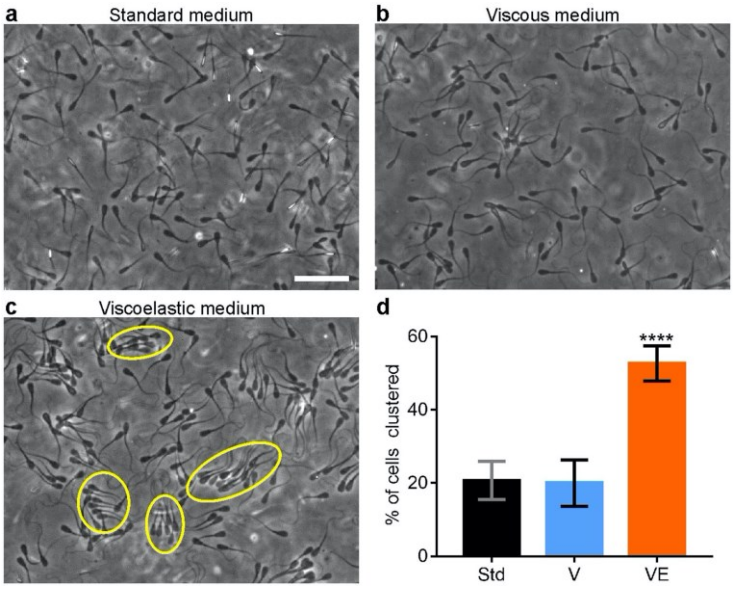

Figure 11. Bull sperm motion in a. standard medium for sperm suspension; b. viscous medium and c. viscoelastic medium. d. Percentage of sperm found in clusters (including pairs) in standard medium (Std), Newtonian viscous medium (V), and viscoelastic medium (VE).

All these studies had provided fundamental basis for the characterization of semen viscosity in steady-state conditions and of spermatozoa motion in a complex medium. Although the rheological behavior in steady-state conditions can help to elucidate the mechanisms governing the non- Newtonian, shear-thinning and viscoplastic behavior of human semen, the response under oscillatory flow could be especially relevant to investigate semen microstructure in the small deformation regime. In fact, the study of semen rheology could play a key role both for a deeper understanding of the effect of semen

physical properties on men fertility and human reproduction, and for bioengineering problems, such as semen storage [179], and design of sperm analysis and selection devices [8]. However, despite all the efforts devoted to this issue until now, a comprehensive, quantitative rheological characterization of human semen viscoelasticity are quite scarce and sparse in the literature, and investigations under oscillatory flow are, to the best of our knowledge, still lacking in the literature, as well as a correlation between semen elasticity and sperm motility.

Aim of the thesis

In this PhD thesis, the first systematic experimental investigation of human semen transient behavior in the linear viscoelastic regime by performing oscillatory shear measurements os provided. The storage (G′) and loss (G″) moduli of human semen have been measured over an extended range of frequencies [0.1–30 rad/s] by using a rotational rheometer. Results show that G″ predominates over G′ across the entire range of frequency explored, with a proximity of the moduli at low frequencies. Moreover, a correlation between sperm motility and semen viscoelastic properties is provided. These findings could help in elucidating the significance of elasticity on the characterization of human semen physical properties, in addition to viscosity, fundamental in pathological situations, where hyperviscosity (and probably elasticity) can be found.

2. Materials and Methods

2.1. Semen samples

58 human semen samples were collected during routine tests from men attending the Sterility Center of the Polyclinic of the University of Naples Federico II, and analyzed by World Health Organization (WHO) criteria [28]. The resulting report, called *spermiogram*, contains information from two types of tests: macroscopic and microscopic, as reported in *Table II*, regarding a semen specimen as normal when its values were above the lower reference limit. Only liquefied samples were considered. According to these criteria, 30 samples were considered as normospermic, and were taken as control.

Due to the small amounts available for each sample (~3 ml), single tests were performed for each donor. All the samples were used as such and stored at room temperature.

Table II: WHO reference values used for the selection of control sampls [28].

Type of examination	**Parameter** (unit)	**Lower reference limit** (range)
Macroscopic	pH	7.2
	viscosity	Length of the tread < 2 cm
Microscopic	Sperm concentration (sperm $*10^6$/mL)	15 (12–16)
	Total motility (PR+NP %)	40 (38 – 42)
	Sperm morphology (normal form %)	4 (3 – 4)

2.2. Rheological measurements

A rheometer dedicated to measurements on biological fluids must meet requirements such as reduced quantities, high sensitivity, temperature control, sample evaporation, and the ease of cleaning parts in contact with biological materials. For the analysis of seminal fluid, a stress-controlled Physica rheometer MCR 301 (Anton-Paar, Graz, Austria) *Figure 12* equipped with a titanium double-gap measuring system (DG 26.7, inner cup diameter 24.267 mm, inner bob diameter 24.666 mm, outer bob diameter 26.663 mm, outer cup diameter 27.053 mm, bob height 40 mm) has been exploited. This assembly ensures high surface area and requires small amount of sample (i.e., 1.8 ml), fundamental for a low viscous fluid and available in small quantities, as semen is. All measurements were performed within 4-6 h from collection, keeping the temperature constant at 25 °C, thanks

the use of a Peltier system. Oil was used to cover the semen surface exposed to air to avoid drying.

Steady shear measurements were performed in the range 2 - 1000 s^{-1}, from low to high shear rates and back (up-down curve), providing viscosity *vs* shear rate curves with 30 measuring data points and sampling time of 10 s.

Semen storage modulus G′ and loss modulus G″ as a function of frequency have been evaluated by oscillatory shear measurements from 30 to 0.1 rad/s and strain amplitude from 1% to 6%, depending on the data provided by amplitude test. In fact, in order to determine the linear viscoelastic regime, each oscillatory test has been preceded by strain amplitude sweep tests at 1 rad/s and 10 rad/s for strain amplitudes from 0.1 to 10 %.

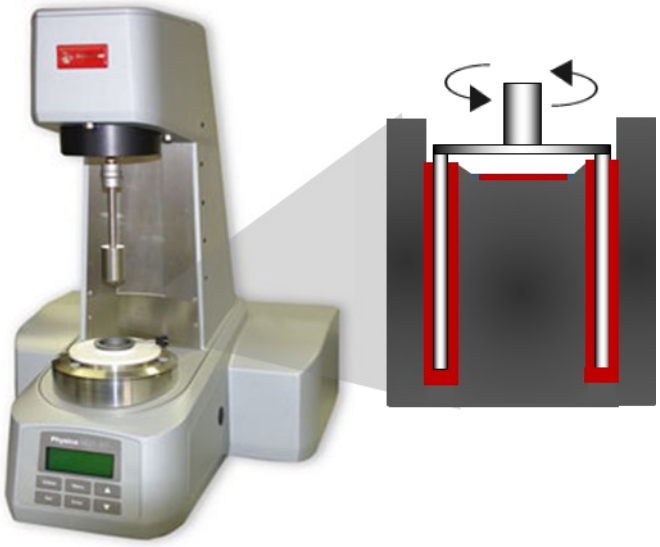

Figure 12. Rotational Viscometer Anton Paar, MCR 301 (Physica) for rheological measurements of seminal fluid.

2.3. Sperm motility analysis

The seminal fluid (about 10 µl) was placed on a slide arranged on the table of an inverted optical microscope (Axio Observer Z1, Zeiss). Images of spermatozoa movement were recorded by using a high-speed camera (Phantom v711 operated up to 100 frames/s), using high-magnification immersion objectives (40x and 100x) in different fields of view for each sample. The so captured images were processed off-line by using a commercial software for image analysis (Image Pro-plus). To evaluate the trajectory of each individual cell of a sample a manual procedure has been followed. In particular, the tracking procedure consisted in the following steps: i) selection of a spermatozoa in the image at some time

point, ii) finding its subsequent positions manually frame by frame (by clicking the mouse on cell center, the cell is marked by a cross-hair in the image overlay to facilitate the identification of the next position), iii) storage of cell x and y coordinates, iv) subtraction of the first value of both x and y coordinates from all the values at subsequent times, to obtain a common origin for all the cells analyzed on a x vs y plot, v) repetition of the procedure for all the cells in the field of view.

3. Results

3.1. Normospermic samples

In the PhD research activity, 58 seminal fluid samples provided by the Sterility Center of the Polyclinic of the University of Naples Federico II have been analyzed. Rheological measurements and characterization of motility were compared to the spermiograms provided by the same center.

30 out of the 58 samples analyzed can be considered as normospermic according to the criteria reported in *Table II*. All the samples have been characterized by rheological measurements, both in steady state and oscillatory tests, as well as by microscopy analysis. In *Figure 13* the flow curves (i.e., viscosity *vs* shear rate) of all the normospermic samples are reported. The viscosity variation of each sample was explored in a range of shear rate between 2 and 1000 1/s. It can be noticed that viscosity decreases with shear rate, in line with the pseudoplastic behavior already noted in literature [19], where it was observed that the difference between the rheological properties of the "normal" and the hyperviscous semen indicates the presence of a complex structure in the seminal plasma.

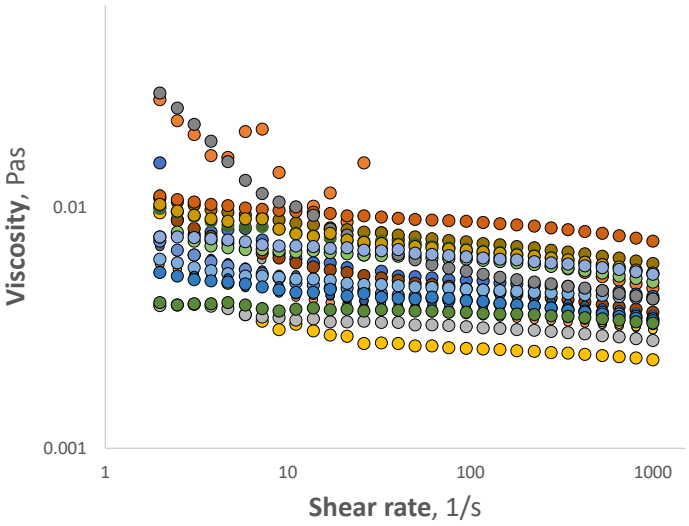

Figure 13. Flow curves of all the normospermic samples.

In *Figure 14a* the mean viscosity of the normospermic samples is reported, confirming a shear thinning behavior, and suggesting a microstructure buildup at low shear rates, which is partially lost at high shear rates. In *Figure 14b*, the linear trend of shear stress as a function

of shear rate is reported, showing very good agreement with previous data from the literature [164].

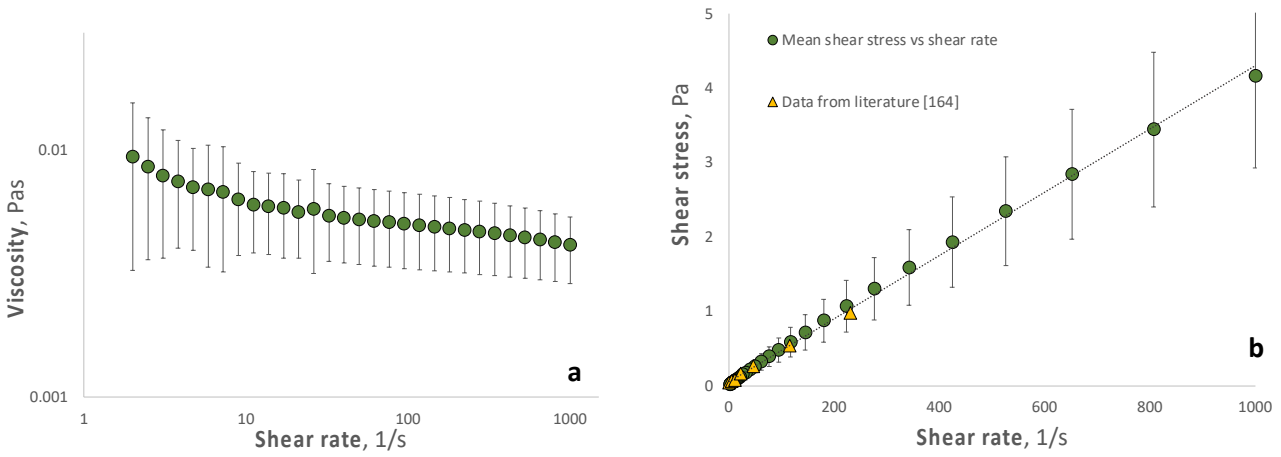

Figure 14: a. Mean Viscosity of normospermic samples as a function of shear rate; b. Mean shear stress as a function of shear rate compared with data from the literature [164].

To test the stability of the sample and to exclude sedimentation, each measure has been carried out by increasing and subsequently decreasing the imposed shear rate without reloading the sample, which allowed to check the absence of hysteresis (as show in *Figure 15*, left, for a reference sample).

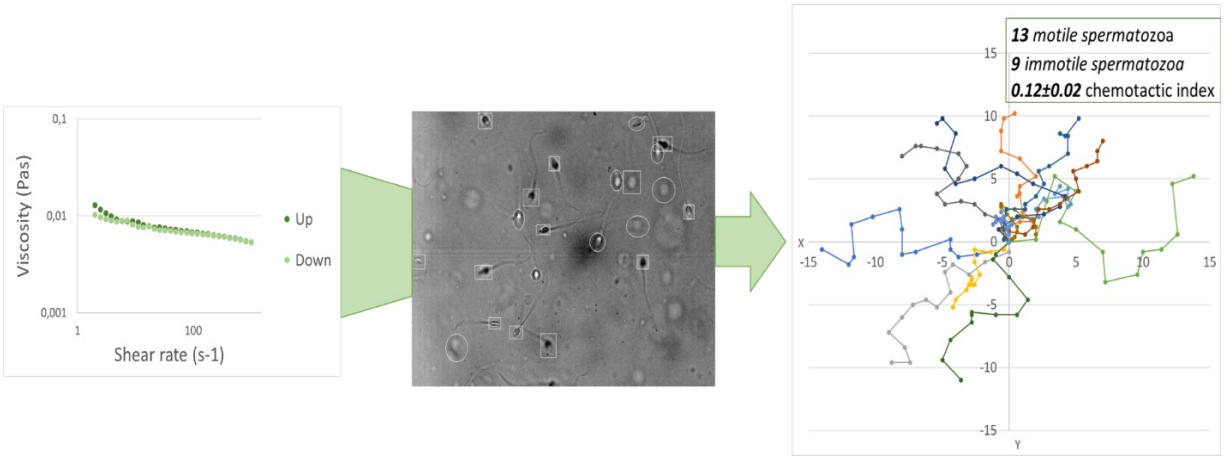

Figure 15. Rheological and microscopic measurements of the reference sample. The graph on the left side represents viscosity curve as a function of shear rate from 1 to 100 s -1. Graph on the right side represents the trajectory of each sperm detected in the sample analyzed with the microscope.

For each sample, images of the movement of spermatozoa were acquired as well, as reported in the picture in the center of *Figure 15*. It is possible to observe (graph on the right of *Figure 15*) that each spermatozoa is characterized by a random distribution of

orientations, without having a preferential direction of motion, as expected for an isotropic sample.

A directional movement parameter is given by the chemotactic index of the kth cell $\boldsymbol{I}_k$

$$\boldsymbol{I}_k = \frac{\sqrt{(x_f - x_0)^2 + (y_f - y_0)^2}}{\sum\sqrt{(x_i - x_0)^2 + (y_i - y_0)^2}}$$

where x_0 and y_0 are the cell coordinates at the beginning of the tracking, x_f and y_f are the cell coordinates of the last tracked position, and x_i and y_i are the cell coordinates along the trajectory. The chemotactic index is equal to 0 for a random trajectory and to 1 for a fully extended directional motion (a straight line). The average value of $\boldsymbol{I}_k$ over the N cells investigated is the overall chemotactic index $\boldsymbol{I}$

$$\boldsymbol{I} = \sum I_k/N$$

As an example, I =0,12 for the reference sample reported in *Figure 15*. The microscopy analysis of sperm motility is in agreement with the data from the spermiogram.

Based on the literature study carried out in this work, while semen viscosity has been measured in previous studies, data on the elastic G′ and viscous G′′ moduli of semen have not been published before in the literature and represent an original contribution of this thesis. Oscillatory shear tests have been performed to evaluate G′ and G′′. Each oscillatory measurement has been preceded by strain amplitude sweep tests at 1 rad/s and 10 rad/s for strain amplitudes from 0.1 to 10%, to determine the linear viscoelastic regime. A strain ranging from 1% to 10%, depending on the data obtained by an amplitude test, has been used in the experiments. A representative plot of an amplitude sweep is shown in *Figure 16*.

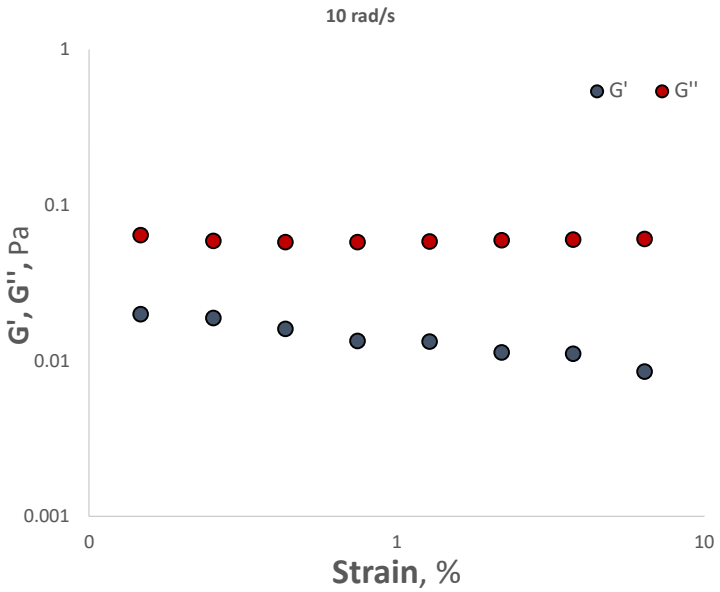

Figure 16. G′ and G″ as a function of frequency at 10 rad/s for a representative normospermic sample.

The frequency sweep tests were performed from 30 to 0.1 rad/s, as shown in *Figure 17*, where G′ and G′′ are plotted as a function of frequency ω for all the normospermic samples.

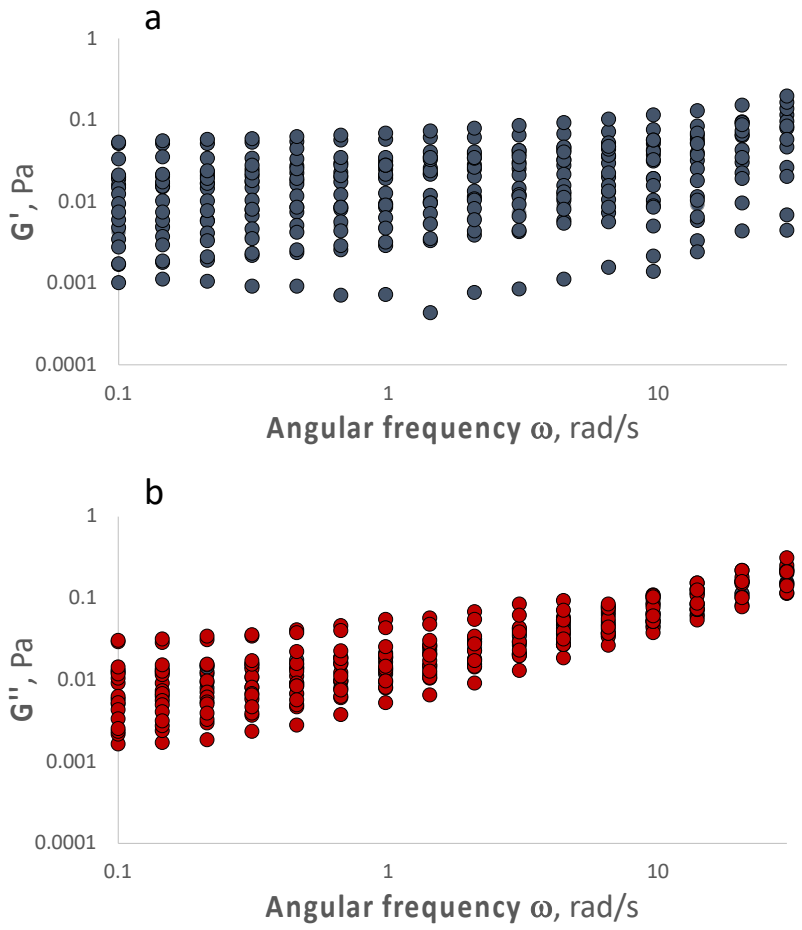

Figure 17: a. G ' and b. G′′ as a function of frequency for all the normospermic samples.

Due to their different behavior (e.g. data show G′-G′′ crossover at different values of frequency or do not show it at all), all the data have been scaled onto a single master curve. The latter has been obtained by independently scaling both the modulus and the frequency of each data set, by factors a and b, respectively, as reported in [180]. In particular, frequency data have been normalized by the crossover frequency (i.e. a= crossover frequency), and the two moduli have been normalized by the plateau value of the G′ (i.e. b= G′ plateau value). The resultant master curve for G′ and G′′ is shown in *Figure 18*. At low scaled frequencies, scaled G′ overcomes G′′ and has nearly no frequency dependence, allowing us to identify a plateau modulus. At intermediate frequency, G′ and G′′ cross. Finally, at high frequencies, G′′ dominates G′ and asymptotically approaches an almost linear dependence on frequency. The overall shape of the scaled data is representative of weakly attractive suspensions. In the inset of *Figure 18*, the plot of the modulus scale factor b as a function of the frequency scale factor a divided by high shear rate viscosity is reported, and a linear relationship can be observed.

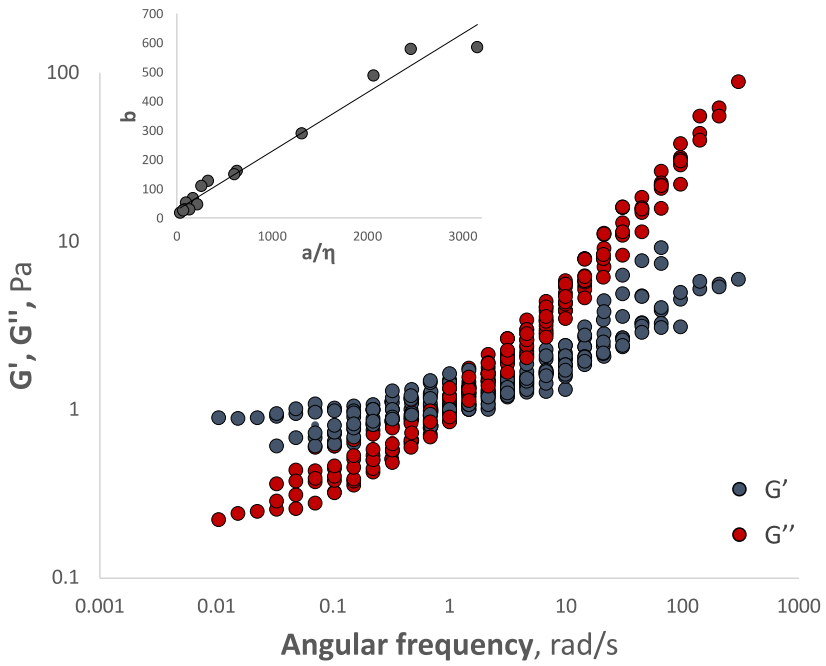

Figure 18. Master curve of G′ and G′′ as a function of frequency. Inset: scaling factor b as a function of scaling factor a divided by high shear rate viscosity.

Oscillatory and steady shear measurements can be compared to each other to assess whether the so-called Cox-Merz rule is valid. According to such rule, the modulus of the complex viscosity $\boldsymbol{\eta}^*(\omega)$ is equal to the steady shear viscosity $\boldsymbol{\eta}(\dot{\boldsymbol{\gamma}})$ when the angular frequency, measured in rad/s, is equal to the steady shear rate, measured in s^{-1}:

$$\boldsymbol{\eta}(\dot{\boldsymbol{\gamma}}) = \boldsymbol{\eta}^*(\omega)$$

The Cox-Merz rule holds for flexible polymers, while it usually fails for fluids with a microstructure dependent on the applied deformation, such as heterogeneous media (e.g., suspensions) and associating polymers.

In *Figure 19 a)* G'' has been plotted as a function of G' for two representative semen samples. The two sets of data lie on curves of different slopes, indicating that the rheological behavior of the samples, in terms of microstructure, is different, even if the spermiogram data of the two samples are quite similar, in terms of sperm motility, concentration, aggregation and pH. In *Figure 19 b)* and *c)* the viscosity and the magnitude of complex viscosity are plotted as a function of shear rate and angular frequency for the same two representative samples. For the sample in *Figure 19 b)* the two sets of data show a good agreement, suggesting a weak elastic behavior or the absence of sperm aggregates. On the other hand, for the sample reported in *Figure 19 c)*, an evident gap between steady state and oscillatory sets of data is found in the entire range of shear rates/frequencies, with complex viscosity values higher than viscosity ones.

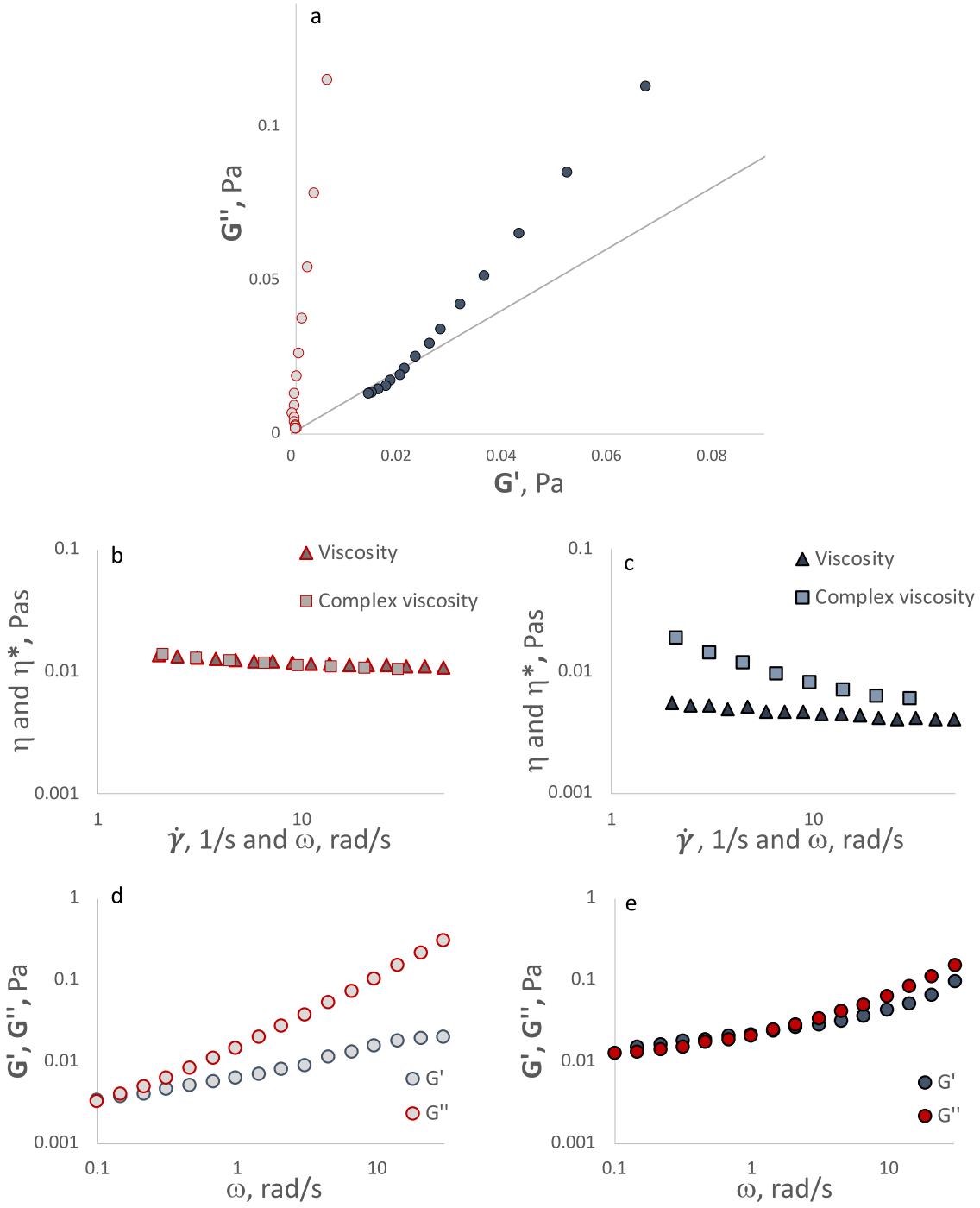

Figure 19. Comparison of rheological response of two representative samples of liquid liquid-like and elastic like-behavior, solid line representing the bisector line. a. G'' vs G'; b. and c. Comparison of viscosity (triangles) and modulus of complex viscosity (squares) by applying the Cox- Merz rule; d. and e. mechanical spectra.

This trend can be related to the presence of aggregated microstructures, which are broken down in steady shear flow, but are preserved under small amplitude oscillatory flow. The frequency spectra of G′ and G″ for the same two samples are shown in *Figure 19 d)* and *e)*

and are characterized by higher values of G" as compared to G'. At low frequency G' and G" tend to overlap with each other and to become almost horizontal, thus showing a gel-like behavior in such frequency range.

Ultimately, the trend of the motility parameters and number of spermatozoa per ml of all the normospermic samples was evaluated as a function of the rheological parameters (G', G" and viscosity) to verify if and how sperm motility and concentration are influenced by or affect rheological properties. In particular, for viscosity 4 values of shear rate (i.e. 2, 10, 100, and 1000 s^{-1}) representative of the whole flow curve have been considered, while for the moduli G' and G" 3 values of frequency (i.e. 0.1, 1 and 10 rad/s) have been considered. The steady state measurements, carried out on 30 normospermic semen samples, have been clustered in 3 groups to evaluate possible effects of sperm concentration on semen viscosity (*Figure 20a*) and the effect of semen viscosity on sperm motility (*Figure 20b*).

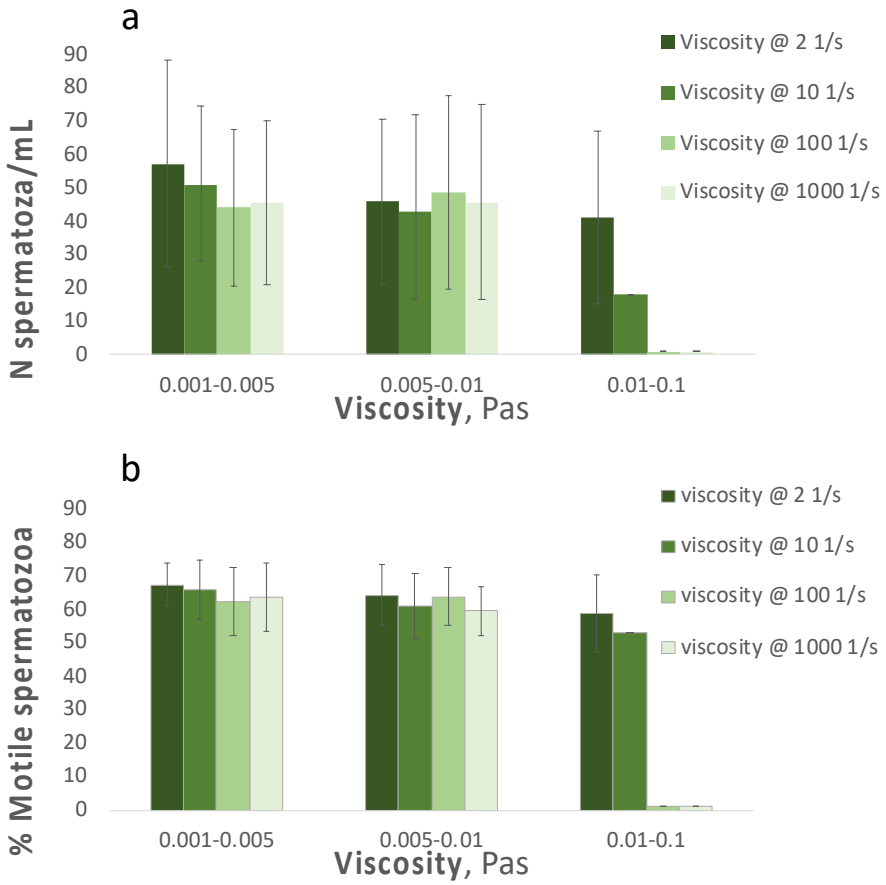

Figure 20: a. Effect of sperm concentration, reported in terms of the number of spermatozoa per mL on semen viscosity; b. sperm motility as a function of semen viscosity. The error bars represent standard deviation.

1. $0.001 < \eta < 0.005$
2. $0.005 < \eta < 0.01$

3. $0.01 < \eta < 0.1$

It seems that there is not any correlation between sperm concentration and semen viscosity nor between sperm motility and semen viscosity.

Regarding oscillatory measurements, the 20 samples analyzed have been clustered in two groups based on moduli values, as follows:

1. 0,001 <G', G''< 0,01
2. 0,01 <G', G''< 0,1

In *Figure 21*, the two upper graphs are referred to G', and report the number of spermatozoa per ml and the percentage of motile spermatozoa and as a function on semen viscosity per 3 values of angular frequency. In particular, *Figure 21a* shows that there is not any correlation between the number of spermatozoa per mL and G, while in *Figure 21b*, a clear increasing trend of the percentage of motile spermatozoa with G' is found, with statistical significative difference ($p<0.01$ with T-test) between the data at high frequency. On the other hand, in the graphs in the bottom of *Figure 21* reporting data referred to G'' any correlation between sperm concentration (*Figure 21c*) nor motility (*Figure 21d*) with semen viscosity is observable.

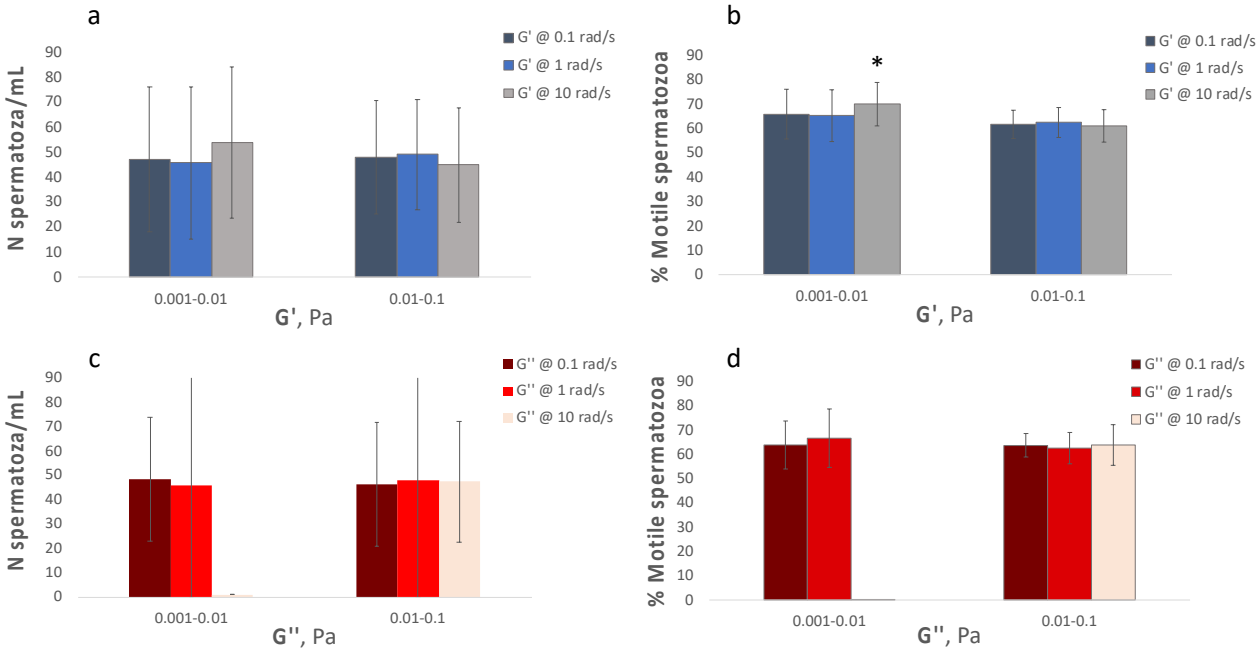

Figure 21: a. sperm concentration and b. sperm motility as a function of G'; c. sperm concentration and d. sperm motility as a function of G''. The error bars represent standard deviations.

3.2. Altered samples

28 out of the 58 seminal fluid samples provided by the Sterility Center of the Polyclinic of the University of Naples Federico II can be considered altered samples (non normospermic) according to WHO guidelines, considering the reference spermiogram. As for the control ones, these samples were subjected to rheological measurements, both in steady state and oscillatory shear flow, and to microscopy analysis. *Figure 22* shows the flow curve of all altered samples. The viscosity variation of the sample was explored in a range of shear rate between 2 and 1000 1/s. The pseudoplastic behavior found in normospermic samples is present also in altered semen samples.

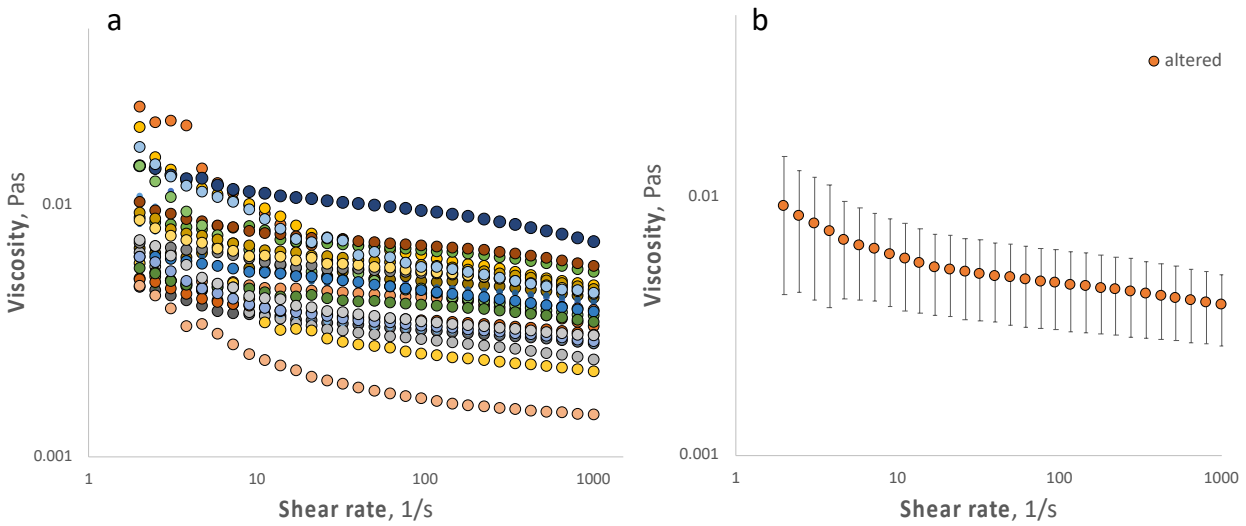

Figure 22: a. Flow curves of all the altered samples; b. mean values of viscosity of altered samples. The error bars represent the standard deviation.

Following the measurement of the flow curve, the elastic G' and viscous G" modulus were measured for all altered samples as a function of frequency. The tests were carried out in a frequency range between 30 and 0.1 rad/s, as shown in *Figure 23.*

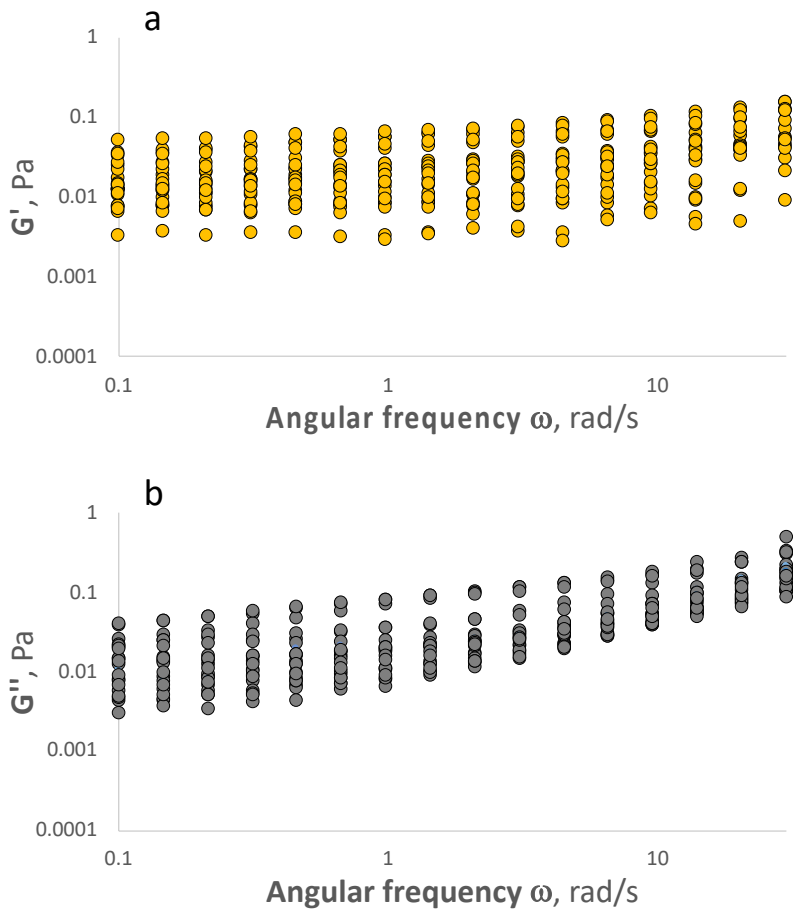

Figure 23: a. G ' and b. G'' as a function of frequency for all the altered samples.

Also in this case, the trend of the motility parameters and number of spermatozoa per ml was evaluated as a function of the rheological parameters (G', G") to verify if and how the two parameters are influenced by a variation of the elastic modulus and of the viscous modulus, as shown in *Figure 24.*

In particular, for G' and G" a low (0.1 rad/s), medium (1 rad/s) and a high (10 rad/s) frequency was considered.

The 28 samples identified were then clustered as follows:

- for the elastic (G ') and viscous (G ") moduli, 2 groups of samples were identified:
    1. $0.001<G', G''< 0.01$
    2. $G', G''< 0.01$

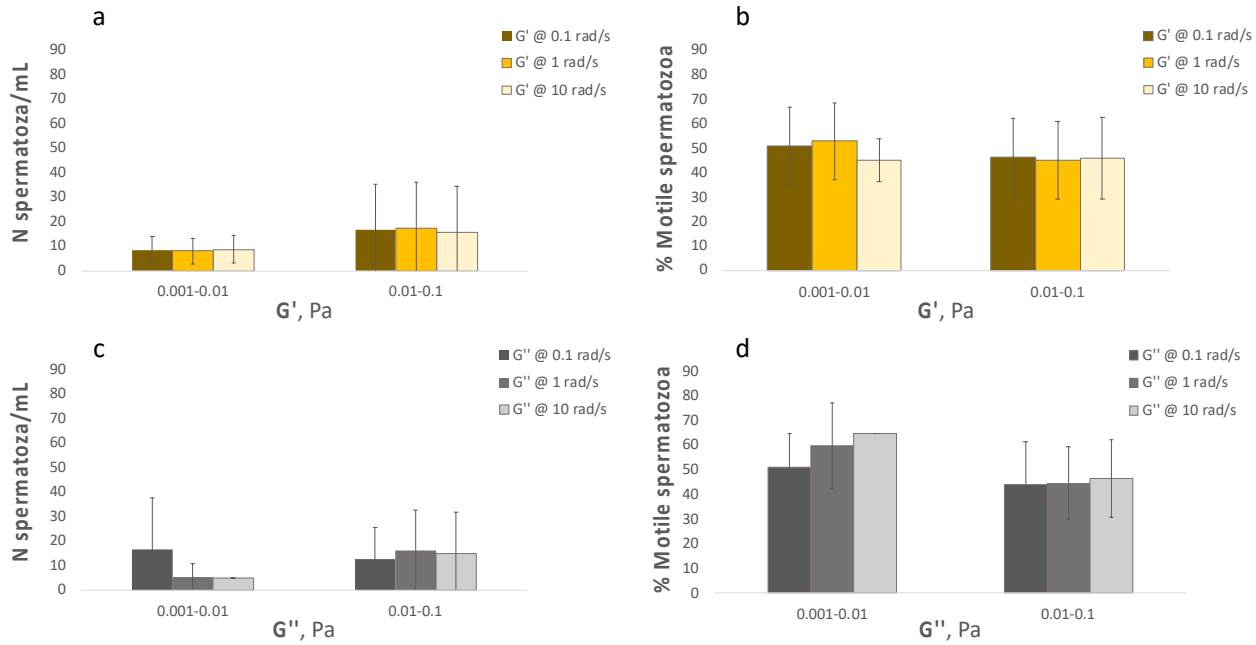

Figure 24: a. sperm concentration and b. sperm motility as a function of G'; c. sperm concentration and d. sperm motility as a function of G''. The error bars represent standard deviations.

It can be seen that for the altered samples, there is not any correlation between the number of spermatozoa per mL and bot G' and G''. Similarly for sperm motility.

3.3. Comparisons between control and altered semen samples

The seminal fluid samples investigated were subjected to rheological (viscosity, elastic modulus and viscous modulus) and motility measurements. Analyzing the spermiograms, the samples were classified into normospermic and altered, on the basis of the parameters expressed by the WHO guidelines. For both groups of samples, the viscosity was analyzed as a function of the shear rate: the viscosity decreases with shear rate, in line with the pseudoplastic behavior; a shear thinning trend is also highlighted for the two set of date (i.e. normospermic and altered) (see *Figure 25*).

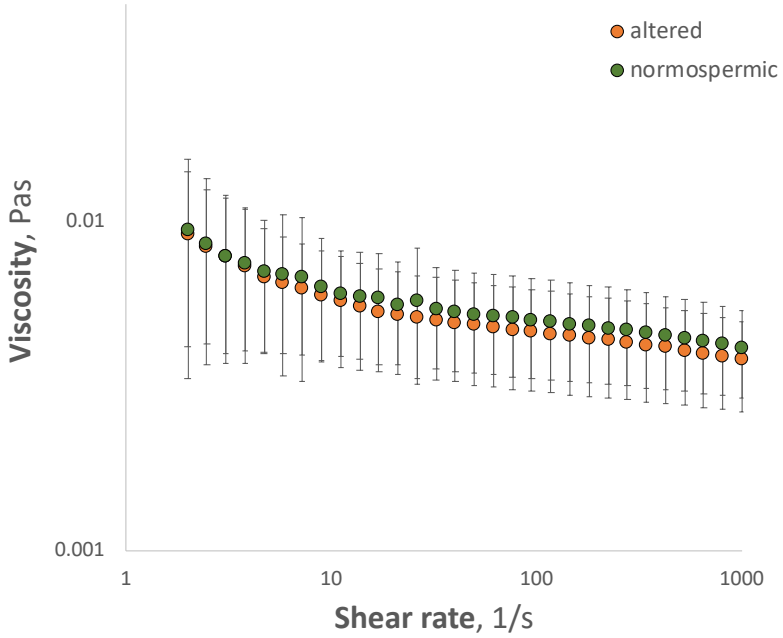

Figure 25. Comparison of normospermic and alterd samples. Green circles represent normospermic samples, orange circles represent altered samples.

The normospermic and altered samples were also compared in terms of the possible relation between G', G'' and sperm concentration and motility. The graphs in *Figure 26* show that for both moduli, motility and spermatozoa concentration values are higher for normospermic samples as compared to altered ones (for the sake of brevity, only data at 1 rads/s are reported). This confirms the clinical hypotheses according to which normospermic samples are actually characterized by greater motility and a greater number of spermatozoa.

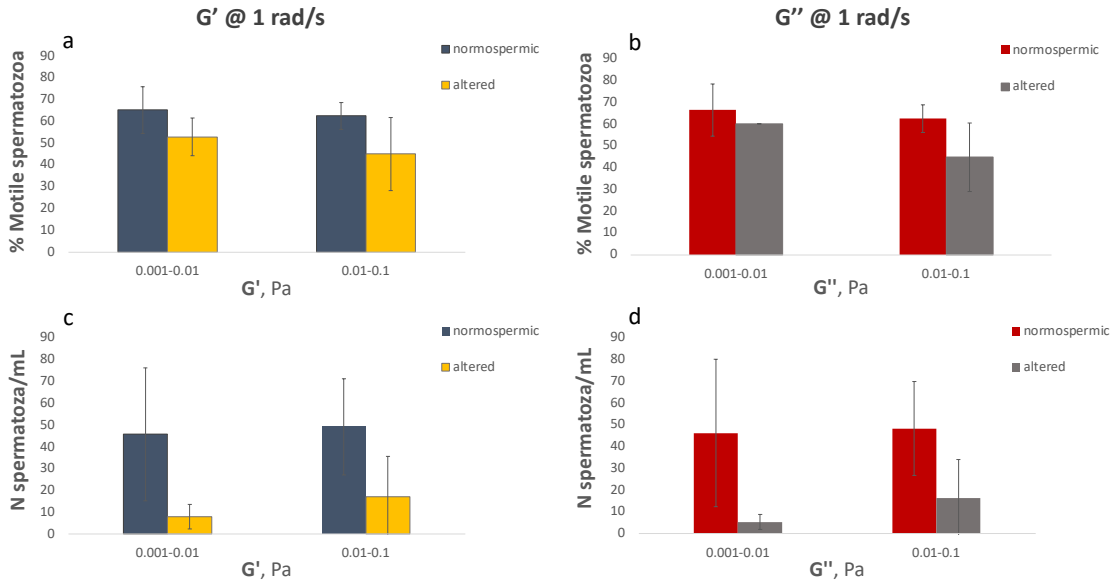

Figure 26: a. sperm concentration and b. sperm motility as a function of G' at 1 rad/s; c. sperm concentration and d. sperm motility as a function of G''at 1 rad/s. The error bars represent standard deviations.

Finally, we selected some samples from the spermiograms provided by the fertility center:

- 5 samples from patients with varicocele (having undergone surgery or not).
- 3 samples evaluated in the fertility laboratory with an increased viscosity.

From a visual analysis it can be observed that the samples classified as ones of increased viscosity or with previous varicocele according to the spermiogram fall within the viscosity range of the samples that do not show particular anomalies (gray points), contrary to what it would have been expected on the basis of the laboratory analyses of the spermiogram (see *Figure 27).*

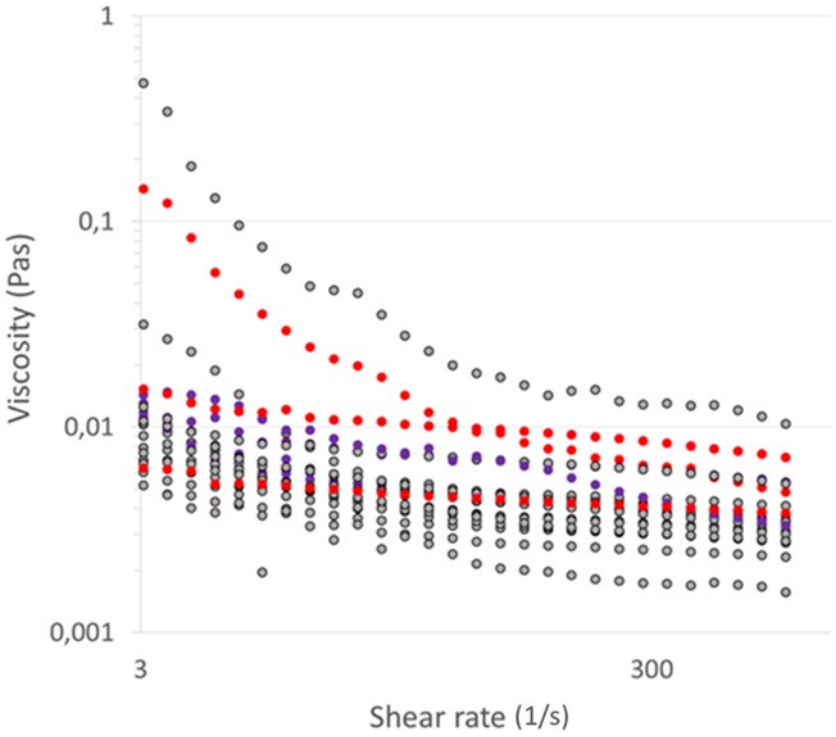

Figure 27. Viscosity assessment of all normospermic and non-normospermic samples. In purple, samples from patients with varicocele; in red the samples with a high viscosity according to the fertility laboratory.

These results confirm that the qualitative assessment of viscosity from the spermiogram is not consistent with the quantitative data provided by rheological tests. This is likely due to the fact that the length of the semen thread, which is taken as a proxy of viscosity in the spermiogram analysis, is also affected by the elastic properties of the semen sample. Therefore, the viscoelastic response in terms of the viscous and elastic moduli provide a more accurate characterization of the semen rheological behavior.

Conclusions

Male infertility is defined as idiopathic when it makes it impossible to lead to pregnancy despite the fact that semen is characterized by a spermiogram with values comprised in the reference ranges. The present PhD work therefore had the objective of analyzing cases of idiopathic infertility by exploring rheological parameters so far not analyzed in the clinical practice, to verify possible discrepancies between normospermic and altered samples (according to the classification obtained by spermiograms).

Following an in-depth literature study, it emerged that the rheological measurements of seminal fluid are limited to the exploration of viscosity (as a function of shear rate) analyzed with different types of viscometer. Our study confirmed what has been highlighted in the literature regarding the characterization of semen as a non-Newtonian fluid with pseudoplastic behavior. Based on the literature study carried out in this work, data on elastic G' and viscous G'' moduli of semen are not present in the literature and represent an original contribution of this thesis. From the measurements carried out, it emerges that semen rheological behavior depends on frequency, showing a master curve typical of weakly attractive suspensions. The comparison between normospermic samples and altered samples, as a function of G', G'' and viscosity, confirms that motility and the number of spermatozoa are higher in normospermic samples compared to altered samples, independently on the frequency value.

As a final analysis, on the basis of the evidence emerging from the spermiograms, samples with previous varicocele and high viscosity were considered; a rheological analysis of viscosity was then carried out for these samples, highlighting that the relative viscosity curves are in the same distribution range as normospermic samples. This confirms that the qualitative methods currently used in fertility centers to evaluate semen rheological response are not reliable.

The purpose of this thesis, therefore, is to add an important piece to the male infertility puzzle by proposing that knowledge of the rheological properties of seminal fluid can provide an additional method for its diagnosis and inspire advances in artificial insemination and medical treatments. Thus, the attention of further research should be directed towards a systematic quantitative assessment of the rheological characteristics of

human seminal fluid, in terms of viscosity and elasticity, defining a quantitative index of semen viscoelasticity to be used in the diagnosis routes, coupled to sperm motility tests. Such an approach could eventually inspire researchers and companies to develop cheap, fast and user-friendly tools for point of care analysis, for example based on microfluidic techniques[8]. Furthemore, quantitative semen viscoelasticity and sperm motility analysis should be associated with genetic tests, which are important infertility factors, as discussed in the Introduction.

References

[1] M. Ikawa, N. Inoue, A.M. Benham, M. Okabe, Fertilization: a sperm's journey to and interaction with the oocyte, The Journal of clinical investigation 120(4) (2010) 984-994.
[2] E.A. Gaffney, H. Gadêlha, D. Smith, J. Blake, J. Kirkman-Brown, Mammalian sperm motility: observation and theory, Annual Review of Fluid Mechanics 43 (2011) 501-528.
[3] S. Gurunath, Z. Pandian, R.A. Anderson, S. Bhattacharya, Defining infertility—a systematic review of prevalence studies, Human reproduction update 17(5) (2011) 575-588.
[4] M.C. Inhorn, P. Patrizio, Infertility around the globe: new thinking on gender, reproductive technologies and global movements in the 21st century, Human reproduction update 21(4) (2015) 411-426.
[5] H. Sun, T.-T. Gong, Y.-T. Jiang, S. Zhang, Y.-H. Zhao, Q.-J. Wu, Global, regional, and national prevalence and disability-adjusted life-years for infertility in 195 countries and territories, 1990–2017: results from a global burden of disease study, 2017, Aging (Albany NY) 11(23) (2019) 10952.
[6] J. Boivin, L. Bunting, J.A. Collins, K.G. Nygren, International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care, Human reproduction 22(6) (2007) 1506-1512.
[7] A. Agarwal, A. Mulgund, A. Hamada, M.R. Chyatte, A unique view on male infertility around the globe, Reproductive biology and endocrinology 13(1) (2015) 37.
[8] R. Nosrati, P.J. Graham, B. Zhang, J. Riordon, A. Lagunov, T.G. Hannam, C. Escobedo, K. Jarvi, D. Sinton, Microfluidics for sperm analysis and selection, Nature Reviews Urology 14(12) (2017) 707-730.
[9] https://ourworldindata.org/fertility-rate.
[10] T. Noda, M. Ikawa, Physiological function of seminal vesicle secretions on male fecundity, Reproductive medicine and biology 18(3) (2019) 241-246.
[11] H. Rodríguez-Martínez, U. Kvist, J. Ernerudh, L. Sanz, J.J. Calvete, Seminal plasma proteins: what role do they play?, American journal of reproductive immunology 66 (2011) 11-22.
[12] B. Macanovic, M. Vucetic, A. Jankovic, A. Stancic, B. Buzadzic, E. Garalejic, A. Korac, B. Korac, V. Otasevic, Correlation between sperm parameters and protein expression of antioxidative defense enzymes in seminal plasma: a pilot study, Disease markers 2015 (2015).
[13] E.F. Schisterman, S.L. Mumford, Z. Chen, R.W. Browne, D. Boyd Barr, S. Kim, G. Buck Louis, Lipid concentrations and semen quality: the LIFE study, Andrology 2(3) (2014) 408-415.
[14] S. BERGSTRÖM, L.A. Carlson, J.R. Weeks, The prostaglandins: a family of biologically active lipids, Pharmacological reviews 20(1) (1968) 1-48.
[15] B. Moreno-Escallon, A. Ridley, C.H. Wu, L. Blasco, Hormones in seminal plasma, Archives of andrology 9(2) (1982) 127-134.
[16] T. Mann, Fructose, a constituent of semen, Nature 157(3977) (1946) 79-79.
[17] G. Humphrey, T. Mann, Citric acid in semen, Nature 161(4088) (1948) 352-353.
[18] M. Nouri, Z.A. GHASEM, L. Farzadi, V. Shahnazi, N.M. GHAFARI, Vitamins C, E and lipid peroxidation levels in sperm and seminal plasma of asthenoteratozoospermic and normozoospermic men, (2008).
[19] N.S. Juyena, C. Stelletta, Seminal plasma: an essential attribute to spermatozoa, Journal of androlog y 33(4) (2012) 536-551.
[20] C. De Jonge, Biological basis for human capacitation, Human Reproduction Update 11(3) (2005) 205-214.
[21] S. Ahmadi, R. Bashiri, A. Ghadiri-Anari, A. Nadjarzadeh, Antioxidant supplements and semen parameters: An evidence based review, International Journal of Reproductive BioMedicine 14(12) (2016) 729.

[22] U. Kvist, Importance of spermatozoal zinc as temporary inhibitor of sperm nuclear chromatin decondensation ability in man, Acta Physiologica Scandinavica 109(1) (1980) 79-84.
[23] N. Okamura, Y. Tajima, H. Ishikawa, S. Yoshii, K. Koiso, Y. Sugita, Lowered levels of bicarbonate in seminal plasma cause the poor sperm motility in human infertile patients, Fertility and sterility 45(2) (1986) 265-272.
[24] A.S. Patel, J.Y. Leong, R. Ramasamy, Prediction of male infertility by the World Health Organization laboratory manual for assessment of semen analysis: A systematic review, Arab journal of urology 16(1) (2018) 96-102.
[25] A. Hamada, S.C. Esteves, M. Nizza, A. Agarwal, Unexplained male infertility: diagnosis and management, International braz j urol 38(5) (2012) 576-594.
[26] J. Zhou, L. Chen, J. Li, H. Li, Z. Hong, M. Xie, S. Chen, B. Yao, The semen pH affects sperm motility and capacitation, PloS one 10(7) (2015) e0132974.
[27] C.W. Macosko, Rheology: Principles, Measurements, and Applications, Wiley1994.
[28] O.-H.T. años del Grupo, World Health Organization manual for the processing of human semen-2010, actas urol esp 34(7) (2010) 577-578.
[29] G.K. Berger, L.I. Smith-Harrison, J.I. Sandlow, Sperm agglutination: Prevalence and contributory factors, Andrologia 51(5) (2019) e13254.
[30] C.F.S. Jensen, P. Østergren, J.M. Dupree, D.A. Ohl, J. Sønksen, M. Fode, Varicocele and male infertility, Nature Reviews Urology 14(9) (2017) 523-533.
[31] K.L.f. O'brien, A.C. Varghese, A. Agarwal, The genetic causes of male factor infertility: a review, Fertility and sterility 93(1) (2010) 1-12.
[32] C. Krausz, A. Riera-Escamilla, Genetics of male infertility, Nature Reviews Urology 15(6) (2018) 369-384.
[33] J.P. Jarow, Effects of varicocele on male fertility, Human reproduction update 7(1) (2001) 59-64.
[34] J. Macleod, Further observations on the role of varicocele in human male infertility, Fertility and sterility 20(4) (1969) 545.
[35] W.H. Organization, The influence of varicocele on parameters of fertility in a large group of men presenting to infertility clinics, Fertility and Sterility 57(6) (1992) 1289-1293.
[36] A. Agarwal, R. Sharma, A. Harlev, S.C. Esteves, Effect of varicocele on semen characteristics according to the new 2010 World Health Organization criteria: a systematic review and meta-analysis, Asian journal of andrology 18(2) (2016) 163.
[37] Y.-J. Wang, R.-Q. Zhang, Y.-J. Lin, R.-G. Zhang, W.-L. Zhang, Relationship between varicocele and sperm DNA damage and the effect of varicocele repair: a meta-analysis, Reproductive biomedicine online 25(3) (2012) 307-314.
[38] M. Roque, S.C. Esteves, Effect of varicocele repair on sperm DNA fragmentation: a review, International urology and nephrology 50(4) (2018) 583-603.
[39] R.A. Saleh, A. Agarwal, R.K. Sharma, T.M. Said, S.C. Sikka, A.J. Thomas Jr, Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele, Fertility and sterility 80(6) (2003) 1431-1436.
[40] F. Comhaire, A. Vermeulen, Varicocele sterility: cortisol and catecholamines, Fertility and sterility 25(1) (1974) 88-95.
[41] M.S. Cohen, L. Plaine, J.S. Brown, The role of internal spermatic vein plasma catecholamine determinations in subfertile men with varicoceles, Fertility and Sterility 26(12) (1975) 1243-1249.
[42] L. Saalu, The incriminating role of reactive oxygen species in idiopathic male infertility: an evidence based evaluation, Pakistan Journal of Biological Sciences 13(9) (2010) 413.

[43] R. Aitken, K. West, D. Buckingham, Leukocytic infiltration into the human ejaculate and its association with semen quality, oxidative stress, and sperm function, Journal of andrology 15(4) (1994) 343-352.
[44] P. Sabeti, S. Pourmasumi, T. Rahiminia, F. Akyash, A.R. Talebi, Etiologies of sperm oxidative stress, International Journal of Reproductive BioMedicine 14(4) (2016) 231.
[45] A. Bui, R. Sharma, R. Henkel, A. Agarwal, Reactive oxygen species impact on sperm DNA and its role in male infertility, Andrologia 50(8) (2018) e13012.
[46] H.M. SHEN, S.E. Chia, C.N. Ong, Evaluation of oxidative DNA damage in human sperm and its association with male infertility, Journal of andrology 20(6) (1999) 718-723.
[47] G. Barroso, M. Morshedi, S. Oehninger, Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa, Human reproduction 15(6) (2000) 1338-1344.
[48] A. Sansone, C. Di Dato, C. de Angelis, D. Menafra, C. Pozza, R. Pivonello, A. Isidori, D. Gianfrilli, Smoke, alcohol and drug addiction and male fertility, Reproductive biology and endocrinology 16(1) (2018) 1-11.
[49] D. Carrell, L. Liu, C. Peterson, K. Jones, H. Hatasaka, L. Erickson, B. Campbell, Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss, Archives of andrology 49(1) (2003) 49-55.
[50] A. Agarwal, N. Parekh, M.K. Panner Selvam, R. Henkel, R. Shah, S.T. Homa, R. Ramasamy, E. Ko, K. Tremellen, S. Esteves, Male oxidative stress infertility (MOSI): proposed terminology and clinical practice guidelines for management of idiopathic male infertility, The world journal of men's health 37(3) (2019) 296-312.
[51] A. Agarwal, R.A. Saleh, M.A. Bedaiwy, Role of reactive oxygen species in the pathophysiology of human reproduction, Fertility and sterility 79(4) (2003) 829-843.
[52] N.K. Duru, M. Morshedi, S. Oehninger, Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa, Fertility and sterility 74(6) (2000) 1200-1207.
[53] P. May-Panloup, M.F. Chrétien, F. Savagner, C. Vasseur, M. Jean, Y. Malthiery, P. Reynier, Increased sperm mitochondrial DNA content in male infertility, Human Reproduction 18(3) (2003) 550-556.
[54] S. Casagrande, M. Hau, Telomere attrition: metabolic regulation and signalling function?, Biology letters 15(3) (2019) 20180885.
[55] J. Thilagavathi, M. Kumar, S. Mishra, S. Venkatesh, R. Kumar, R. Dada, Analysis of sperm telomere length in men with idiopathic infertility, Archives of gynecology and obstetrics 287(4) (2013) 803-807.
[56] A. Agarwal, E. Tvrda, R. Sharma, Relationship amongst teratozoospermia, seminal oxidative stress and male infertility, Reproductive Biology and Endocrinology 12(1) (2014) 1-8.
[57] I. Alkan, F. Simsek, G. Haklar, E. Kervancioglu, H. Ozveri, S. Yalcin, A. Akdas, Reactive oxygen species production by the spermatozoa of patients with idiopathic infertility: relationship to seminal plasma antioxidants, The Journal of urology 157(1) (1997) 140-143.
[58] F. Pasqualotto, R. Sharma, H. Kobayashi, D. Nelson, A. Agarwal, Oxidative stress in normospermic men undergoing infertility evaluation, Journal of andrology 22(2) (2001) 316-322.
[59] S.E. Lewis, P.M. Boyle, K.A. McKinney, I.S. Young, W. Thompson, Total antioxidant capacity of seminal plasma is different in fertile and infertile men, Fertility and sterility 64(4) (1995) 868-870.
[60] A. Pilatz, H. Hossain, R. Kaiser, A. Mankertz, C.G. Schuettler, E. Domann, H.-C. Schuppe, T. Chakraborty, W. Weidner, F. Wagenlehner, Acute epididymitis revisited: impact of molecular diagnostics on etiology and contemporary guideline recommendations, European urology 68(3) (2015) 428-435.

[61] H.C. Schuppe, A. Meinhardt, J. Allam, M. Bergmann, W. Weidner, G. Haidl, Chronic orchitis: a neglected cause of male infertility?, Andrologia 40(2) (2008) 84-91.
[62] A. Azenabor, A.O. Ekun, O. Akinloye, Impact of inflammation on male reproductive tract, Journal of reproduction & infertility 16(3) (2015) 123.
[63] V. Syriou, D. Papanikolaou, A. Kozyraki, D.G. Goulis, Cytokines and male infertility, European cytokine network 29(3) (2018) 73-82.
[64] K.L. Loveland, B. Klein, D. Pueschl, S. Indumathy, M. Bergmann, B.E. Loveland, M.P. Hedger, H.-C. Schuppe, Cytokines in male fertility and reproductive pathologies: immunoregulation and beyond, Frontiers in Endocrinology 8 (2017) 307.
[65] J.G. Alvarez, R.K. Sharma, M. Ollero, R.A. Saleh, M.C. Lopez, A.J. Thomas Jr, D.P. Evenson, A. Agarwal, Increased DNA damage in sperm from leukocytospermic semen samples as determined by the sperm chromatin structure assay, Fertility and sterility 78(2) (2002) 319-329.
[66] G.R. Dohle, Male infertility in cancer patients: review of the literature, International journal of urology 17(4) (2010) 327-331.
[67] G. Cavallini, G. Beretta, Clinical management of male infertility, Springer2015.
[68] W.H.B. Wallace, R.A. Anderson, D.S. Irvine, Fertility preservation for young patients with cancer: who is at risk and what can be offered?, The lancet oncology 6(4) (2005) 209-218.
[69] D. Kumar, S.R. Salian, G. Kalthur, S. Uppangala, S. Kumari, S. Challapalli, S.G. Chandraguthi, H. Krishnamurthy, N. Jain, P. Kumar, Semen abnormalities, sperm DNA damage and global hypermethylation in health workers occupationally exposed to ionizing radiation, PLoS One 8(7) (2013) e69927.
[70] D. Chan, G. Delbès, M. Landry, B. Robaire, J.M. Trasler, Epigenetic alterations in sperm DNA associated with testicular cancer treatment, Toxicological Sciences 125(2) (2012) 532-543.
[71] N.J. van Casteren, G.H. van der Linden, F.G. Hakvoort-Cammel, K. Hählen, G.R. Dohle, M.M. van den Heuvel-Eibrink, Effect of childhood cancer treatment on fertility markers in adult male long-term survivors, Pediatric blood & cancer 52(1) (2009) 108-112.
[72] H. Kobayashi, K. Larson, R.K. Sharma, D.R. Nelson, D.P. Evenson, H. Toma, A.J. Thomas Jr, A. Agarwal, DNA damage in patients with untreated cancer as measured by the sperm chromatin structure assay, Fertility and sterility 75(3) (2001) 469-475.
[73] A. Yoshida, K. Miura, M. Shirai, Cytogenetic Survey of 1,007 Infertile Males, The Journal of Urology 160(5) (1998) 1941-1941.
[74] M.M. Arafa, A. Majzoub, S.S. AlSaid, W. El Ansari, A. Al Ansari, Y. Elbardisi, H.T. Elbardisi, Chromosomal abnormalities in infertile men with azoospermia and severe oligozoospermia in Qatar and their association with sperm retrieval intracytoplasmic sperm injection outcomes, Arab journal of urology 16(1) (2018) 132-139.
[75] M. Shamsi, K. Kumar, R. Dada, Genetic and epigenetic factors: Role in male infertility, Indian journal of urology: IJU: journal of the Urological Society of India 27(1) (2011) 110.
[76] V. D'Argenio, F. Cariati, R. Tomaiuolo, One4Two®: An Integrated Molecular Approach to Optimize Infertile Couples' Journey, Genes 12(1) (2021) 60.
[77] J. Poongothai, T. Gopenath, S. Manonayaki, Genetics of human male infertility, Singapore Med J 50(4) (2009) 336-347.
[78] M.S. Oud, L. Volozonoka, R.M. Smits, L.E. Vissers, L. Ramos, J.A. Veltman, A systematic review and standardized clinical validity assessment of male infertility genes, Human Reproduction 34(5) (2019) 932-941.
[79] D. Plaseska-Karanfilska, P. Noveski, T. Plaseski, I. Maleva, S. Madjunkova, Z. Moneva, Genetic causes of male infertility, Balkan Journal of Medical Genetics 15(Supplement) (2012) 31-34.
[80] T. Huynh, R. Mollard, A. Trounson, Selected genetic factors associated with male infertility, Human Reproduction Update 8(2) (2002) 183-198.

[81] R. Flannigan, P.N. Schlegel, Genetic diagnostics of male infertility in clinical practice, Best Practice & Research Clinical Obstetrics & Gynaecology 44 (2017) 26-37.
[82] F. Lanfranco, A. Kamischke, M. Zitzmann, E. Nieschlag, Klinefelter's syndrome, The Lancet 364(9430) (2004) 273-283.
[83] N.A. Deebel, A.W. Bradshaw, H. Sadri-Ardekani, Infertility considerations in klinefelter syndrome: From origin to management, Best Practice & Research Clinical Endocrinology & Metabolism (2020) 101480.
[84] S.A. Brosman, Mixed Gonadal Dysgenesi, The Journal of urology 121(3) (1979) 344-347.
[85] E. Vorona, M. Zitzmann, J.r. Gromoll, A.N. Schüring, E. Nieschlag, Clinical, endocrinological, and epigenetic features of the 46, XX male syndrome, compared with 47, XXY Klinefelter patients, The Journal of Clinical Endocrinology & Metabolism 92(9) (2007) 3458-3465.
[86] A. Ferlin, F. Raicu, V. Gatta, D. Zuccarello, G. Palka, C. Foresta, Male infertility: role of genetic background, Reproductive biomedicine online 14(6) (2007) 734-745.
[87] C. Krausz, E. Casamonti, Spermatogenic failure and the Y chromosome, Human genetics 136(5) (2017) 637-655.
[88] A.C. Chandley, Chromosome anomalies and Y chromosome microdeletions as causal factors in male infertility, Human Reproduction 13(suppl_1) (1998) 45-50.
[89] P.B. Davis, Cystic Fibrosis, Pediatr Rev 22(8) (2001) 257-264.
[90] V. Dumur, R. Gervais, J.-M. Rigot, E. Delomel-Vinner, B. Decaestecker, J.-J. Lafitte, P. Roussel, Congenital bilateral absence of the vas deferens (CBAVD) and cystic fibrosis transmembrane regulator (CFTR): correlation between genotype and phenotype, Human genetics 97(1) (1996) 7-10.
[91] O. Hiort, P.-M. Holterhus, T. Horter, W. Schulze, B. Kremke, M. Bals-Pratsch, G.H. Sinnecker, K. Kruse, Significance of mutations in the androgen receptor gene in males with idiopathic infertility, The Journal of Clinical Endocrinology & Metabolism 85(8) (2000) 2810-2815.
[92] Y.-Z. Ge, L.-W. Xu, R.-P. Jia, Z. Xu, W.-C. Li, R. Wu, S. Liao, F. Gao, S.-J. Tan, Q. Song, Association of polymorphisms in estrogen receptors (ESR1 and ESR2) with male infertility: a meta-analysis and systematic review, Journal of assisted reproduction and genetics 31(5) (2014) 601-611.
[93] J.D. Ring, A.A. Lwin, T.S. Köhler, Current medical management of endocrine-related male infertility, Asian journal of andrology 18(3) (2016) 357.
[94] C. Dodé, J.-P. Hardelin, Kallmann syndrome, European Journal of Human Genetics 17(2) (2009) 139-146.
[95] C. Krausz, A. Riera-Escamilla, Monogenic forms of male infertility, Genetics of Endocrine Diseases and Syndromes (2019) 341-366.
[96] T. Raivio, J. Falardeau, A. Dwyer, R. Quinton, F.J. Hayes, V.A. Hughes, L.W. Cole, S.H. Pearce, H. Lee, P. Boepple, Reversal of idiopathic hypogonadotropic hypogonadism, New England Journal of Medicine 357(9) (2007) 863-873.
[97] M. Pengo, A. Ferlin, B. Arredi, F. Ganz, R. Selice, A. Garolla, C. Foresta, FSH receptor gene polymorphisms in fertile and infertile Italian men, Reproductive biomedicine online 13(6) (2006) 795-800.
[98] S. Goodwin, J.D. McPherson, W.R. McCombie, Coming of age: ten years of next-generation sequencing technologies, Nature Reviews Genetics 17(6) (2016) 333.
[99] P. Braude, S. Pickering, F. Flinter, C.M. Ogilvie, Preimplantation genetic diagnosis, Nature Reviews Genetics 3(12) (2002) 941-953.
[100] K. Sermon, A. Van Steirteghem, I. Liebaers, Preimplantation genetic diagnosis, The Lancet 363(9421) (2004) 1633-1641.
[101] C. Carnegie, Diagnosis of hypogonadism: clinical assessments and laboratory tests, Reviews in Urology 6(Suppl 6) (2004) S3.
[102] Y.C. Chooi, C. Ding, F. Magkos, The epidemiology of obesity, Metabolism 92 (2019) 6-10.

[103] J.E. Chavarro, T.L. Toth, D.L. Wright, J.D. Meeker, R. Hauser, Body mass index in relation to semen quality, sperm DNA integrity, and serum reproductive hormone levels among men attending an infertility clinic, Fertility and sterility 93(7) (2010) 2222-2231.
[104] H.I. Kort, J.B. Massey, C.W. Elsner, D. Mitchell-Leef, D.B. Shapiro, M.A. Witt, W.E. Roudebush, Impact of body mass index values on sperm quantity and quality, Journal of andrology 27(3) (2006) 450-452.
[105] A. Katib, Mechanisms linking obesity to male infertility, Central European journal of urology 68(1) (2015) 79.
[106] A. Tchernof, J.-P. Després, A. Bélanger, A. Dupont, D. Prud'homme, S. Moorjani, P.J. Lupien, F. Labrie, Reduced testosterone and adrenal C19 steroid levels in obese men, Metabolism 44(4) (1995) 513-519.
[107] P.N. Schlegel, Aromatase inhibitors for male infertility, Fertility and sterility 98(6) (2012) 1359-1362.
[108] S.S. Du Plessis, S. Cabler, D.A. McAlister, E. Sabanegh, A. Agarwal, The effect of obesity on sperm disorders and male infertility, Nature Reviews Urology 7(3) (2010) 153.
[109] A.N. Comninos, C.N. Jayasena, W.S. Dhillo, The relationship between gut and adipose hormones, and reproduction, Human reproduction update 20(2) (2014) 153-174.
[110] T.P. Kohn, J.R. Kohn, N.M. Haney, A.W. Pastuszak, L.I. Lipshultz, The effect of sleep on men's health, Translational andrology and urology 9(Suppl 2) (2020) S178.
[111] R. Ivell, Lifestyle impact and the biology of the human scrotum, Reproductive biology and endocrinology 5(1) (2007) 1-8.
[112] P. Thonneau, L. Bujan, L. Multigner, R. Mieusset, Occupational heat exposure and male fertility: a review, Human Reproduction (Oxford, England) 13(8) (1998) 2122-2125.
[113] K. Leisegang, S. Dutta, Do lifestyle practices impede male fertility?, Andrologia 53(1) (2021) e13595.
[114] C. Paul, D.W. Melton, P.T. Saunders, Do heat stress and deficits in DNA repair pathways have a negative impact on male fertility?, MHR: Basic science of reproductive medicine 14(1) (2008) 1-8.
[115] J.H. Kim, H.J. Kim, H.S. Noh, G.S. Roh, S.S. Kang, G.J. Cho, S.K. Park, B.J. Lee, W.S. Choi, Suppression by ethanol of male reproductive activity, Brain research 989(1) (2003) 91-98.
[116] A. Calogero, R. Polosa, A. Perdichizzi, F. Guarino, S. La Vignera, A. Scarfia, E. Fratantonio, R. Condorelli, O. Bonanno, N. Barone, Cigarette smoke extract immobilizes human spermatozoa and induces sperm apoptosis, Reproductive biomedicine online 19(4) (2009) 564-571.
[117] T. Viloria, N. Garrido, J.L. Fernández, J. Remohí, A. Pellicer, M. Meseguer, Sperm selection by swim-up in terms of deoxyribonucleic acid fragmentation as measured by the sperm chromatin dispersion test is altered in heavy smokers, Fertility and sterility 88(2) (2007) 523-525.
[118] H. Trummer, H. Habermann, J. Haas, K. Pummer, The impact of cigarette smoking on human semen parameters and hormones, Human Reproduction 17(6) (2002) 1554-1559.
[119] A. Harlev, A. Agarwal, S.O. Gunes, A. Shetty, S.S. du Plessis, Smoking and male infertility: an evidence-based review, The world journal of men's health 33(3) (2015) 143.
[120] D. Dutta, A. Ramachandran, D.T. Leighton, Effect of channel geometry on solute dispersion in pressure-driven microfluidic systems, Microfluidics and Nanofluidics 2(4) (2006) 275-290.
[121] D.M. Nudell, M.M. Monoski, L.I. Lipshultz, Common medications and drugs: how they affect male fertility, The Urologic clinics of North America 29(4) (2002) 965-973.
[122] V. Alonso, V. Linares, M. Bellés, M.L. Albina, J.J. Sirvent, J.L. Domingo, D.J. Sánchez, Sulfasalazine induced oxidative stress: a possible mechanism of male infertility, Reproductive toxicology 27(1) (2009) 35-40.
[123] C. Wang, C. Lai, K. Lam, K. Yeung, Effect of cimetidine on gonadal function in man, British Journal of Clinical Pharmacology 13(6) (1982) 791-794.

[124] A.J. Hamada, B. Montgomery, A. Agarwal, Male infertility: a critical review of pharmacologic management, Expert opinion on pharmacotherapy 13(17) (2012) 2511-2531.
[125] M. Semet, M. Paci, J. Saïas-Magnan, C. Metzler-Guillemain, R. Boissier, H. Lejeune, J. Perrin, The impact of drugs on male fertility: a review, Andrology 5(4) (2017) 640-663.
[126] B.D. Anawalt, The silent spermatozoon: are man-made endocrine disruptors killing male fertility?, Asian journal of andrology 15(2) (2013) 165.
[127] J. Carré, N. Gatimel, J. Moreau, J. Parinaud, R. Léandri, Does air pollution play a role in infertility?: a systematic review, Environmental Health 16(1) (2017) 1-16.
[128] Y. Ménézo, B. Dale, M. Cohen, DNA damage and repair in human oocytes and embryos: a review, Zygote 18(4) (2010) 357-365.
[129] M. Radwan, J. Jurewicz, K. Polańska, W. Sobala, P. Radwan, M. Bochenek, W. Hanke, Exposure to ambient air pollution-does it affect semen quality and the level of reproductive hormones?, Annals of human biology 43(1) (2016) 50-56.
[130] M. Kim, J. Costello, DNA methylation: an epigenetic mark of cellular memory, Experimental & molecular medicine 49(4) (2017) e322-e322.
[131] B. Jin, Y. Li, K.D. Robertson, DNA methylation: superior or subordinate in the epigenetic hierarchy?, Genes & cancer 2(6) (2011) 607-617.
[132] H. Cedar, Y. Bergman, Linking DNA methylation and histone modification: patterns and paradigms, Nature Reviews Genetics 10(5) (2009) 295-304.
[133] B. Zhang, X. Pan, RDX induces aberrant expression of microRNAs in mouse brain and liver, Environmental health perspectives 117(2) (2009) 231-240.
[134] M.J. Jardim, microRNAs: implications for air pollution research, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 717(1-2) (2011) 38-45.
[135] V. Bollati, B. Marinelli, P. Apostoli, M. Bonzini, F. Nordio, M. Hoxha, V. Pegoraro, V. Motta, L. Tarantini, L. Cantone, Exposure to metal-rich particulate matter modifies the expression of candidate microRNAs in peripheral blood leukocytes, Environmental health perspectives 118(6) (2010) 763-768.
[136] C. Vecoli, L. Montano, M.G. Andreassi, Environmental pollutants: genetic damage and epigenetic changes in male germ cells, Environmental Science and Pollution Research 23(23) (2016) 23339-23348.
[137] N. Kothandaraman, A. Agarwal, M. Abu-Elmagd, M.H. Al-Qahtani, Pathogenic landscape of idiopathic male infertility: new insight towards its regulatory networks, NPJ genomic medicine 1(1) (2016) 1-9.
[138] H. Zhang, Y. Liu, D. Su, Y. Yang, G. Bai, D. Tao, Y. Ma, S. Zhang, A single nucleotide polymorphism in a miR-1302 binding site in CGA increases the risk of idiopathic male infertility, Fertility and sterility 96(1) (2011) 34-39. e7.
[139] A. Agarwal, N. Parekh, M.K.P. Selvam, R. Henkel, R. Shah, S.T. Homa, R. Ramasamy, E. Ko, K. Tremellen, S. Esteves, Male oxidative stress infertility (MOSI): proposed terminology and clinical practice guidelines for management of idiopathic male infertility, The world journal of men's health 37(3) (2019) 296.
[140] B. Yu, Z. Huang, Variations in antioxidant genes and male infertility, BioMed research international 2015 (2015).
[141] M. Das, N. Al-Hathal, M. San-Gabriel, S. Phillips, I.-J. Kadoch, F. Bissonnette, H. Holzer, A. Zini, High prevalence of isolated sperm DNA damage in infertile men with advanced paternal age, Journal of assisted reproduction and genetics 30(6) (2013) 843-848.
[142] Y.H. Edrey, A.B. Salmon, Revisiting an age-old question regarding oxidative stress, Free Radical Biology and Medicine 71 (2014) 368-378.

[143] M. Kimura, N. Itoh, S. Takagi, T. Sasao, A. Takahashi, N. Masumori, T. Tsukamoto, Balance of apoptosis and proliferation of germ cells related to spermatogenesis in aged men, Journal of andrology 24(2) (2003) 185-191.
[144] S.D. Lundy, S.C. Vij, Male infertility in renal failure and transplantation, Translational andrology and urology 8(2) (2019) 173.
[145] S. Holdsworth, R. Atkins, D. De Kretser, The pituitary-testicular axis in men with chronic renal failure, New England Journal of Medicine 296(22) (1977) 1245-1249.
[146] D.J. Hawksworth, A.L. Burnett, Nonalcoholic Fatty Liver Disease, Male Sexual Dysfunction, and Infertility: Common Links, Common Problems, Sexual medicine reviews 8(2) (2020) 274-285.
[147] F. Dimitriadis, G. Adonakis, A. Kaponis, C. Mamoulakis, A. Takenaka, N. Sofikitis, Pre-testicular, testicular, and post-testicular causes of male infertility, Endocrinology of the testis and male reproduction. Cham: Springer (2017) 1-47.
[148] S.S. Du Plessis, S. Gokul, A. Agarwal, Semen hyperviscosity: causes, consequences, and cures, Front Biosci (Elite Ed) 5 (2013) 224-231.
[149] J. Elia, M. Delfino, N. Imbrogno, F. Capogreco, M. Lucarelli, T. Rossi, F. Mazzilli, Human semen hyperviscosity: prevalence, pathogenesis and therapeutic aspects, Asian journal of andrology 11(5) (2009) 609.
[150] M. Lin, T. Tsai, Y. Yang, Measurement of viscosity of human semen with a rotational viscometer, Journal of the Formosan Medical Association 91(4) (1992) 419-423.
[151] N. Esfandiari, H. Burjaq, L. Gotlieb, R.F. Casper, Seminal hyperviscosity is associated with poor outcome of in vitro fertilization and embryo transfer: a prospective study, Fertility and sterility 90(5) (2008) 1739-1743.
[152] G. Tomaiuolo, G. Rusciano, S. Caserta, A. Carciati, V. Carnovale, P. Abete, A. Sasso, S. Guido, A New method to improve the clinical evaluation of cystic fibrosis patients by mucus viscoelastic properties, PloS one 9(1) (2014) e82297.
[153] R. Condorelli, A. Calogero, L. Mongioi, E. Vicari, G. Russo, F. Lanzafame, S. La Vignera, Varicocele and concomitant dilation of the periprostatic venous plexus: effects on semen viscosity sperm parameters, Journal of endocrinological investigation 39(5) (2016) 543-547.
[154] A.B. Harchegani, H. Rahmani, E. Tahmasbpour, A. Shahriary, Hyperviscous semen causes poor sperm quality and male infertility through induction of oxidative stress, Current urology 13(1) (2019) 1-6.
[155] G. Gonzales, G. Kortebani, A. Mazzolli, Hyperviscosity and hypofunction of the seminal vesicles, Archives of andrology 30(1) (1993) 63-68.
[156] L. Siciliano, P. Tarantino, F. Longobardi, V. Rago, C. De Stefano, A. Carpino, Impaired seminal antioxidant capacity in human semen with hyperviscosity or oligoasthenozoospermia, Journal of andrology 22(5) (2001) 798-803.
[157] E.T. Issa Layali, M. Joulaei, S.G.A. Jorsaraei, P. Farzanegi, Total antioxidant capacity and lipid peroxidation in semen of patient with hyperviscosity, Cell Journal (Yakhteh) 16(4) (2015) 554.
[158] D. Tjioe, S. Oentoeng, The viscosity of human semen and the percentage of motile spermatozoa, Fertility and sterility 19(4) (1968) 562-565.
[159] A. Ray, N. Chaudhuri, P. Nag, Inconsistent influence of sperm concentration on the viscosity of human seminal fluid, Indian journal of experimental biology 15(9) (1977) 792-794.
[160] A.G. McDonnell, T.C. Gopesh, J. Lo, M. O'Bryan, L.Y. Yeo, J.R. Friend, R. Prabhakar, Motility induced changes in viscosity of suspensions of swimming microbes in extensional flows, Soft Matter 11(23) (2015) 4658-4668.
[161] P.F. Dunn, B.F. Picologlou, Investigation of the rheological properties of human semen, Biorheology 14(5-6) (1977) 277-292.

[162] P.F. Dunn, B.F. Picologlou, Variation in human semen viscoelastic properties with respect to time post ejaculation and frequency of ejaculation, Int J Fertil 22 (1977) 217-224.
[163] B. Aydemir, I. Onaran, A.R. Kiziler, B. Alici, M.C. Akyolcu, The influence of oxidative damage on viscosity of seminal fluid in infertile men, Journal of andrology 29(1) (2008) 41-46.
[164] G. Mendeluk, F.L.G. Flecha, P.R. CASTELLO, C. BREGNI, Factors involved in the biochemical etiology of human seminal plasma hyperviscosity, Journal of andrology 21(2) (2000) 262-267.
[165] H. Hübner, R. Heidl, D.W. KRAUSE, Investigation of flow behaviour (viscosity) from human seminal fluid with a rotational viscosimeter, Andrologia 17(6) (1985) 592-597.
[166] G. Tomaiuolo, A. Carciati, S. Caserta, S. Guido, Blood linear viscoelasticity by small amplitude oscillatory flow, Rheologica Acta 55(6) (2016) 485-495.
[167] F. Striggow, M. Medina-Sánchez, G.K. Auernhammer, V. Magdanz, B.M. Friedrich, O.G. Schmidt, Sperm-driven micromotors moving in oviduct fluid and viscoelastic media, Small 16(24) (2020) 2000213.
[168] Z. Liu, K. Zhang, X. Cheng, Rheology of bacterial suspensions under confinement, Rheologica Acta 58(8) (2019) 439-451.
[169] J.S. Guasto, J.B. Estrada, F. Menolascina, L.J. Burton, M. Patel, C. Franck, A. Hosoi, R.K. Zimmer, R. Stocker, Flagellar kinematics reveals the role of environment in shaping sperm motility, Journal of the Royal Society Interface 17(170) (2020) 20200525.
[170] D.J. Smith, E. Gaffney, H. Gadêlha, N. Kapur, J. Kirkman-Brown, Bend propagation in the flagella of migrating human sperm, and its modulation by viscosity, Cell motility and the cytoskeleton 66(4) (2009) 220-236.
[171] D. Tam, A. Hosoi, Optimal kinematics and morphologies for spermatozoa, Physical Review E 83(4) (2011) 045303.
[172] J.C. Kirkman-Brown, D.J. Smith, Sperm motility: is viscosity fundamental to progress?, MHR: Basic science of reproductive medicine 17(8) (2011) 539-544.
[173] H.C. Fu, C.W. Wolgemuth, T.R. Powers, Beating patterns of filaments in viscoelastic fluids, Physical Review E 78(4) (2008) 041913.
[174] A. Creppy, O. Praud, X. Druart, P.L. Kohnke, F. Plouraboué, Turbulence of swarming sperm, Physical Review E 92(3) (2015) 032722.
[175] S.S. Suarez, Mammalian sperm interactions with the female reproductive tract, Cell and tissue research 363(1) (2016) 185-194.
[176] J. Teran, L. Fauci, M. Shelley, Viscoelastic fluid response can increase the speed and efficiency of a free swimmer, Physical review letters 104(3) (2010) 038101.
[177] C.-k. Tung, C. Lin, B. Harvey, A.G. Fiore, F. Ardon, M. Wu, S.S. Suarez, Fluid viscoelasticity promotes collective swimming of sperm, Scientific reports 7(1) (2017) 1-9.
[178] A. Creppy, F. Plouraboué, O. Praud, X. Druart, S. Cazin, H. Yu, P. Degond, Symmetry-breaking phase transitions in highly concentrated semen, Journal of The Royal Society Interface 13(123) (2016) 20160575.
[179] A. McEvoy, P. Roberts, K. Yap, P. Matson, Development of a simplified method of human semen storage for the testing of sperm DNA fragmentation using the Halosperm G2 test kit, Fertility and sterility 102(4) (2014) 981-988.
[180] V. Trappe, D. Weitz, Scaling of the viscoelasticity of weakly attractive particles, Physical review letters 85(2) (2000) 449.