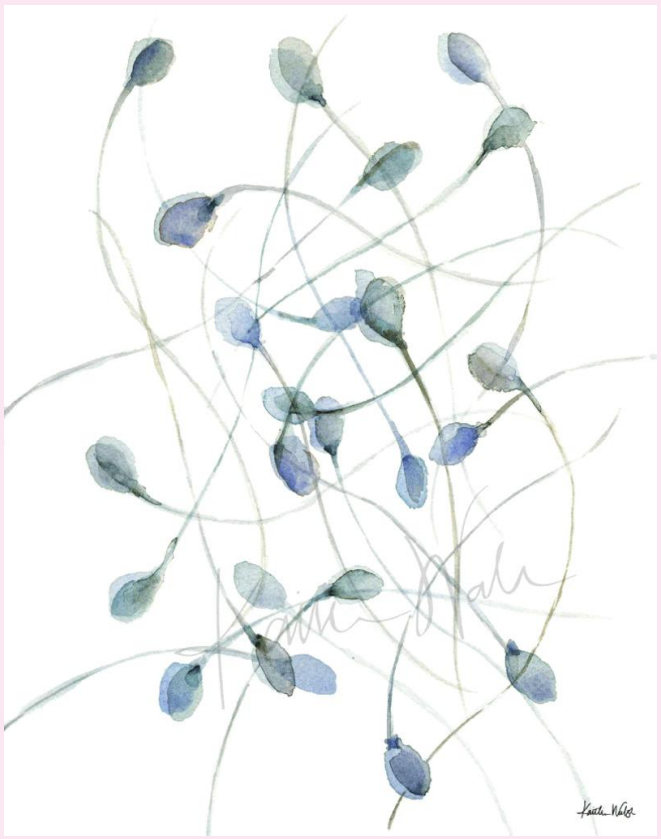
UNIVERSITAT ROVIRA i VIRGILI
Facultat de Química

SARS-CoV-2 INFECTION IMPACT ON MALE REPRODUCTIVE SYSTEM

- A PROSPECTIVE MULTICENTER STUDY -

Final Degree Project

Cristina Torredemer Ortega Tarragona 2024



Sperm in Blue Watercolor © 2017 Lyon Road Art.

Tutor: Dr. Josep Gómez Álvarez
Professional tutor: Dr. Meritxell Jodar Bifet

Grau en Bioquímica i Biologia Molecular - Facultat de Química

This project is based on the results obtained during my external internship with the Molecular Biology of Reproduction and Development Research Group at the University of Barcelona's Faculty of Medicine, under the supervision of Dr. Meritxell Jodar Bifet.

ACKNOWLEDGEMENTS

First of all, I would like to thank myself for the dedication, resilience and countless hours of hard work that have made this project a reality. We both know it has not been an easy journey and despite all you made it, I am proud of you.

Secondly, I would like to express my gratitude to all the people who guided me with their experience and knowledge. Their insightful suggestions have been instrumental in this journey and have guided the direction of my work.

Lastly, these pages are dedicated to my unconditionally support, the people who deeply love me and believed in myself even when I did not. Without you, this would not have been possible.

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ABSTRACT

The significant decline in semen quality and male reproductive health observed over the past 50 years underscores the urgent need for comprehensive research into this public health concern, especially in the context of ongoing and future epidemics caused by new variants of coronaviruses. This study aimed to assess the effects of SARS-CoV-2 infection on semen quality by analysing various parameters in blood and semen samples. Additionally, we compared the results based on severity and the time elapsed since infection.

Our findings confirm that SARS-CoV-2 infection particularly affects seminal parameters and, consequently, male fertility in patients with an active severe infection. However, these parameters appear to improve after one spermatogenic cycle (approximately 90 days), aligning with observations reported in previously published literature.

INTRODUCTION BACKGROUND AND PRE-EXISTING KNOWLEDGE

Before initiating this scientific study, it was essential to review the current knowledge related to our research focus. By synthesizing previous research, we aimed to identify gaps, clarify uncertainties and establish a solid groundwork for our investigation. This section provides an overview of the foundational concepts, key discoveries and ongoing debates in the field that guided our study design and objectives.

MALE INFERTILITY

Infertility, defined as the inability to conceive after a year or more of regular, unprotected sexual intercourse, affects both male and female reproductive systems. It is estimated that approximately 15 to 20% of couples of reproductive age are affected by this condition. The causes of infertility are varied, with around 40% of cases attributed to male factors, another 40% to female factors and the remaining 20% due to a combination of issues from both partners. As a result, male infertility accounts for about half of all infertility cases [1].

A critical step in evaluating male fertility is the semen analysis or seminogram, which assesses semen quality through both macroscopic and microscopic examinations. Macroscopic analysis includes parameters such as colour, volume and viscosity, while microscopic analysis focuses on sperm concentration, motility, morphology and vitality. In some cases, additional tests like hormonal analysis (levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone and inhibin B (INHB)) or DNA fragmentation assessments are conducted.

The guidelines and reference values followed during the semen analysis are the ones included in the latest version of “WHO Laboratory Manual for the Examination and Processing of Human Semen” [2] (Table 1). Various semen abnormalities are categorized according to these values.

A reduced amount of semen or the complete absence of ejaculation is referred to as “hypospermia” or “aspermia,” respectively. When a patient has a sperm concentration below the normal range in their semen, the condition is termed “oligozoospermia.” If the sperm count is 0 million/mL, it is called “azoospermia.” A low percentage of sperm with progressive or overall motility leads to a diagnosis of “asthenozoospermia.” The term “necrozoospermia” is used when there is low sperm vitality, while “teratozoospermia” describes a condition where the percentage of normally shaped sperm is below the standard threshold. These abnormalities do not always occur in isolation; they can appear together, resulting in more complex clinical scenarios. In such cases, the conditions are named by combining the relevant terms, such as “oligoasthenozoospermia” or “asthenoteratozoospermia.”

Table 1. Reference values for semen parameters (lower fifth percentile with 95% confidence interval). *Adapted from World Health Organization, 2021* [2]

	WHO Laboratory Manual - 6th Edition (2021)
Semen volume (mL)	1.4 (1.3-1.5)
Sperm concentration (10^6/mL)	16 (15-18)
Total sperm number (10^6 per ejaculate)	39 (35-40)
Sperm total motility (%)	42 (40-43)
Sperm progressive motility (%)	30 (29-31)
Vitality (%)	54 (50-56)
Normal forms (%)	4 (3.9-4)

Over the past 50 years, a significant decline in semen quality and male reproductive health has been observed, raising serious concerns among healthcare professionals. Current lifestyle choices, increasing exposure to environmental pollution and the impact of various viral pandemics in the 21st century are believed to be key contributors to this troubling trend [3,4]. Extensive research has documented changes in semen parameters, particularly in sperm concentration, among patients infected with different viral strains [5]. This decline became even more pronounced following the outbreak of COVID-19, one of the most significant public health challenges of the last century.

CORONAVIRUSES WITH FOCUS ON SARS-CoV-2

Coronaviruses (CoVs) are enveloped, positive-sense single-stranded RNA (+ssRNA) viruses with a virion size ranging from 80 to 220 nm. They have one of the largest genomes among RNA viruses, encoding a replicase polyprotein and structural proteins, including spike (S-protein), which is essential for the entry of the virus into host cells (**Figure 1**) [6]. RNA viruses, such as CoVs, exhibit a significantly higher evolutionary rate compared to DNA viruses. This is due to their high susceptibility to replication errors mediated by RNA polymerase or reverse transcriptase, coupled with the large size of the viral population and its elevated replication rate [7].

In recent years, three novel coronaviruses have emerged: SARS-CoV, MERS-CoV and SARS-CoV-2, all of which share the ability to replicate in the lower respiratory tract and cause fatal pneumonia. Among these, SARS-CoV-2 is responsible for the outbreak of the Coronavirus disease 2019 (COVID-19 disease), considered one of the biggest emergencies in the history of public health. It marks the third severe epidemic caused by β-CoVs in humans over the past two decades, following the outbreaks of the Severe Acute Respiratory Syndrome (SARS) and the Middle East Respiratory Syndrome (MERS) in 2002 and 2012, respectively [6,7].

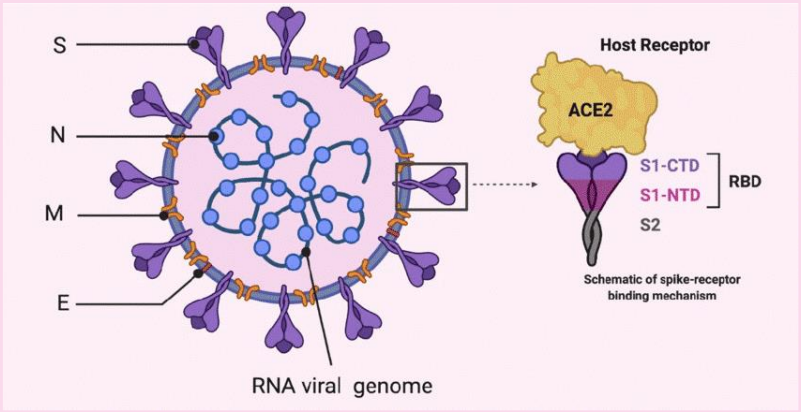


Figure 1. Coronavirus structure showing the organization of spike (S), membrane (M) and envelope (E) proteins. The viral RNA is associated with the nucleocapsid protein (N). ACE2: Angiotensin Converting Enzyme 2; RBD: Receptor-Binding Domain; S1-NTD: N-Terminal Domain; S1-CTD: C-Terminal Domain; S1: amino termini; S2: carboxy termini. *Extracted from Mahmood et al., 2020* [8]

Currently, four typical stages in the development of acute viral infection have been described: invasion; primary blockade of antiviral innate immunity; engagement of the virus's protection mechanisms against the factors of adaptive immunity; and acute, long-term complications of COVID-19. The invasion state starts with the attachment of the S-protein of the virus to the Angiotensin Converting Enzyme 2 (ACE2), a key regulator of the Renin-Angiotensin System (RAS) that serves as the functional receptor of SARS-CoV-2. The S-protein, organized into trimers on the virus surface, undergoes proteolytic cleavage by Transmembrane Serine Protease 2 (TMPRSS2) after the Receptor-Binding Domain (RBD)-receptor interaction. This scission divides the S-protein into the N-terminal S1 subunit and the C-terminal S2 subunit. TMPRSS2 activates the S-protein, enabling membrane fusion between the virus and the target cell, subsequently releasing viral RNA into the cell's cytoplasm (Figure 2 and 3) [7].

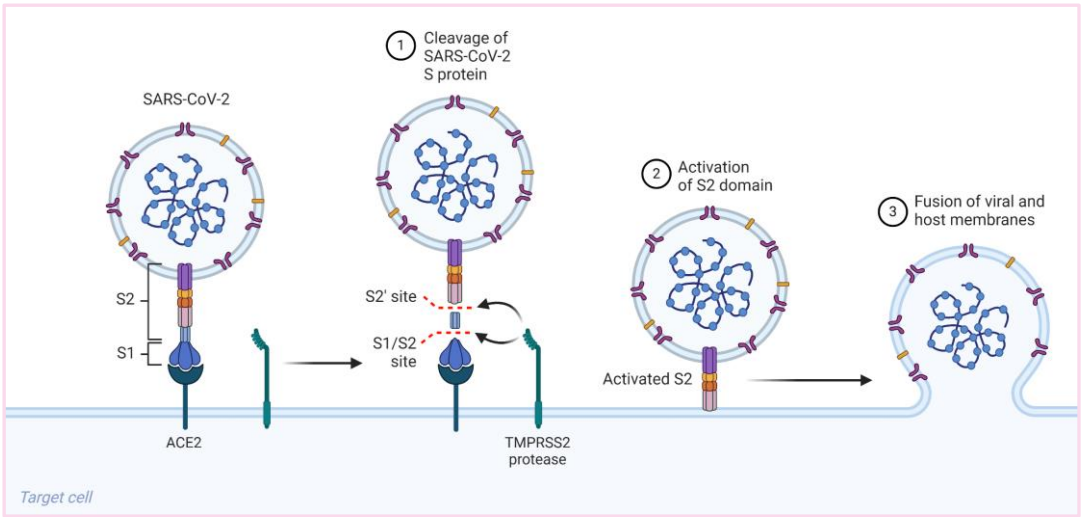


Figure 2. Mechanism of SARS-CoV-2 viral entry. The S2 domain is activated through cleavage by TMPRSS2. TMPRSS2: Transmembrane Serine Protease 2; ACE2: Angiotensin Converting Enzyme 2. *Extracted from Biorender Team, 2020* [9]

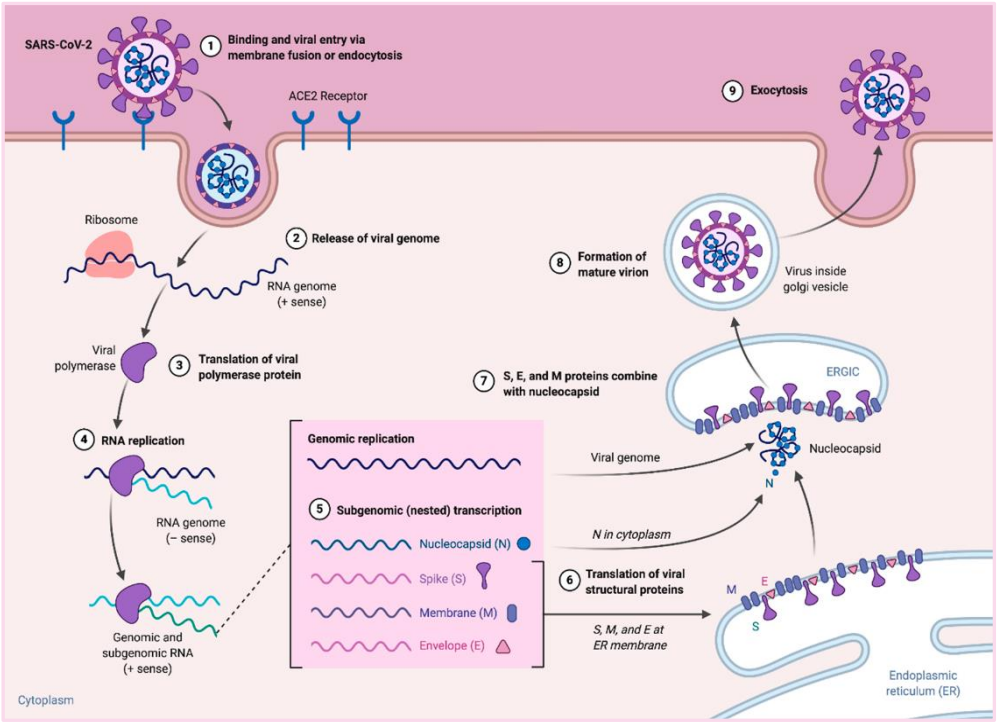


Figure 3. SARS-CoV-2 replication cycle. Several therapeutic targets have been identified in the viral replication steps and are being explored for possible antiviral drug control strategies. *Extracted from Mahmood et al., 2020* [8]

ACE2 AND SARS-CoV-2: IMPLICATIONS FOR REPRODUCTIVE HEALTH

ACE2 is a receptor found on the surface of various cell types, including respiratory epithelial cells, endothelial cells and reproductive system cells, where it plays a crucial role in regulating male fertility [10]. Along with TMPRSS2, ACE2 serves as the primary entry point for SARS-CoV-2 into cells; therefore, the coexpression of these two elements is believed to predict the susceptibility of cells to infection [11].

According to the Human Protein Atlas, ACE2 is primarily located in Sertoli cells, Leydig cells, the epididymis, seminal vesicles and spermatogonia in the male reproductive system [12]. These findings have been corroborated by several studies which, utilizing cell RNA sequencing datasets, demonstrated high expression levels of ACE2 and TMPRSS2 in the testis, including spermatogonia, peritubular myoid cells, testicular somatic cells and spermatogonial stem cells (Figure 4) [13]. This colocalization suggests that testes are highly susceptible to SARS-CoV-2 infection and its associated RAS impairment could potentially impact semen quality [10].

Recent research has identified a diverse range of proteins that can act as ACE2 cofactors or alternative receptors, broadening the spectrum of cell types susceptible to SARS-CoV-2 infection beyond those expressing ACE2 (Table 2) [7].

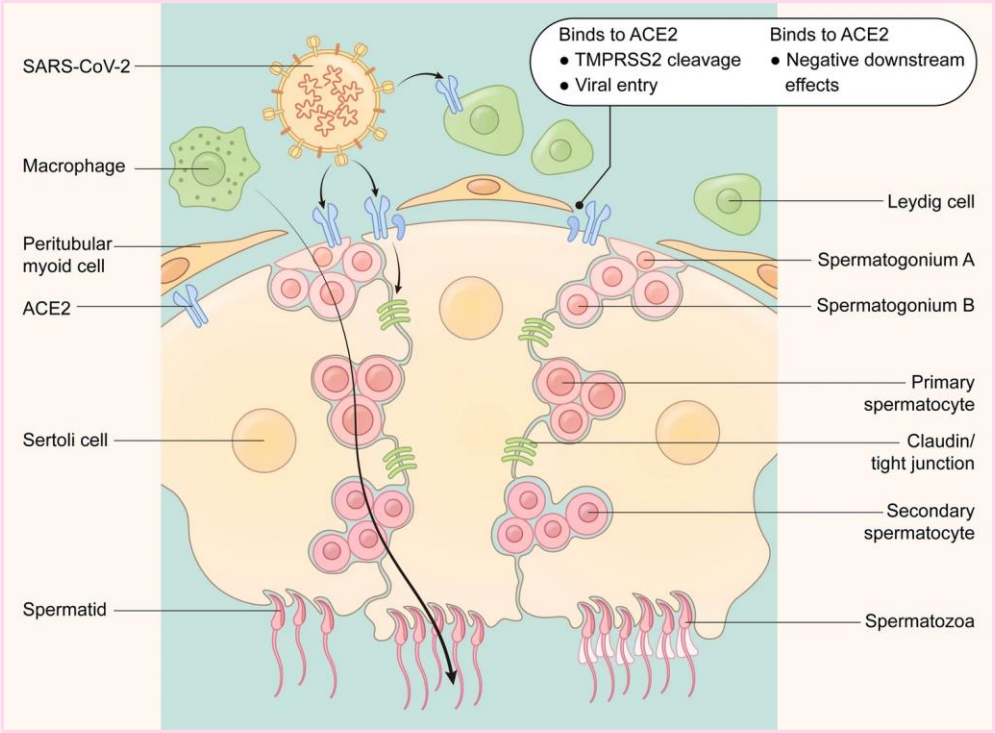


Figure 4. SARS-CoV-2 invades and damages the testes through the widely expressed receptors ACE2 on testicular cells. Various cell types, including Leydig cells, Sertoli cells and germ cells with different developing stages widely express ACE2, which mediates the intrusion of SARS-CoV-2 through the blood–testis barrier together with TMPRSS2. *Extracted from Zhang et al., 2024* [**14**]

Table 2. Some examples of prospective alternative and cofactorial receptors for ACE2 SARS-CoV-2. AXL: tyrosine-protein kinase receptor; ACE2: Angiotensin-Converting Enzyme 2. *Adapted from Gusev et al., 2022* [**7**]

RECEPTOR [Ref]	EXPRESSION ON CELLS
Chondroitin sulfate [15,16]	Most of the cells.
Neuropilin 1 (NRP1, CD304) [17-19]	Nerve cells of the brain and nasal cavity, endothelial cells.
AXL [20]	Expression of AXL > ACE2 in many tissues, and in the lungs and bronchi.
CD147 (Basigin) [21,22]	It is widely expressed in human tissues, highly expressed on cells of the immune system.
GRP78 (BiP, HSPA5) [23-27]	On different cells.

SARS-CoV-2, INFLAMMATION AND OXIDATIVE STRESS

INFLAMMATION

Inflammation can appear during infections like SARS-CoV-2, resulting in excessive cytokine production and cell death [6]. Elevated interleukin 6 (IL-6) levels in critical patients have been closely linked to the serum SARS-CoV-2 viral load and may contribute to acute respiratory distress syndrome (ARDS) development [6,28]. Additionally, autoimmune aggression during SARS-CoV-2 infection involves the breach of biological barriers in immunoprivileged organs (central nervous system, eye, testes and placenta). This breach allows adaptive immunity access to potential autoantigens, exacerbating the impact of SARS-CoV-2 on these vital systems.

The pathogenesis of COVID-19 involves three main inflammatory processes: local classical inflammation, acute systemic inflammation and chronic systemic low-grade inflammation [7]. These inflammatory reactions not only contribute to the severity of COVID-19 but also have long-term implications for patients' health, potentially leading to lasting damage in affected organs and increasing the risk of chronic health conditions.

OXIDATIVE STRESS

A key contributor to inflammation at the molecular level is oxidative stress, which arises from an imbalance in redox homeostasis. This imbalance can result from an increase in reactive species and/or a decrease in cellular antioxidant defenses [29]. While reactive oxygen species (ROS) play a physiological role in sperm function, recent studies have associated abnormally high ROS levels (ranging from 30-80%) in the semen of infertile patients with diminished semen quality [30]. Sperm concentration, motility and DNA integrity are believed to be the seminal parameters most affected by this phenomenon, each showing a tendency to decline as oxidative stress levels increase [29,31].

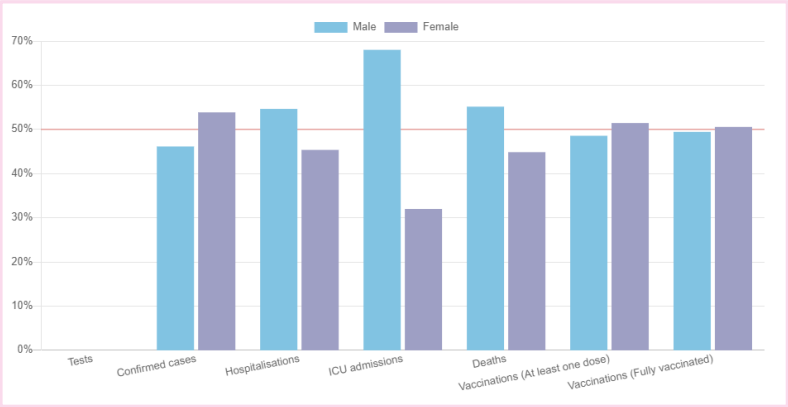
COVID-19: IMPACT ON SPERM PARAMETERS AND TESTOSTERONE LEVELS

Numerous studies have documented changes in sperm quality, including reductions in sperm count and motility, among patients infected by SARS-CoV-2 compared to controls [11,13]. However, it remains unclear whether these alterations arise from direct viral effects or indirect factors such as testicular inflammation. Despite potential impairments in spermatogenesis caused by SARS-CoV-2 infection, evidence suggests that these effects typically resolve within approximately one spermatogenic cycle (approximately 3 months in humans) [32].

Beyond the impact on sperm quality, there’s also a bidirectional effect between SARS-CoV-2 and hormones. Elevated testosterone levels, typically found in men compared to women, may play a crucial role in increasing vulnerability of males to severe COVID-19. It has been suggested that this hormone can facilitate the viral entry into host cells by upregulating the expression of both ACE2 and TMPRSS2 while inducing an immunosuppressive effect that could exacerbate the severity of COVID-19 (**Figure 5**) [13].

Despite physiological levels of testosterone potentially predisposing individuals to COVID-19, increasing evidence suggests that testosterone secretion is impaired during the acute phase of COVID-19, with levels dropping during the infection but rising during the recovery phase.
Studies have identified significant correlations between serum testosterone levels, inflammatory markers, disease progression and clinical outcome, however, the lack of long-term data has complicated reaching a definitive conclusion [13].

A



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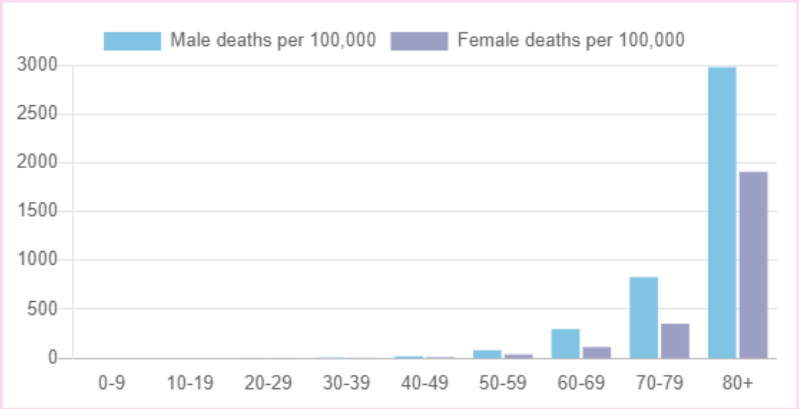


Figure 5A and 5B. A: Comparison of COVID-19 statistics by sex. B: COVID-19 death rates by age and sex. Men tend to experience more severe COVID-19 infections, leading to higher rates of hospitalization and death compared to women. Although these graphs are based on data from Spain, they mirror global trends. *Extracted from Global Health 50/50* [33]

PRESENCE OF SARS-CoV-2 RNA IN SEMEN

The exceptionally hard protective outer shell of SARS-CoV-2 suggests that the virus may be resilient in bodily fluids such as saliva and semen [7]. Previous research on the presence of SARS-CoV-2 RNA in semen across different stages of COVID-19 has given mixed results. Some studies report no detection of viral RNA, while others identify the virus in semen during both acute and recovery phases, though infrequently [13,14]. The presence of viral particles in semen due to urinary shedding remains a possibility that cannot be completely dismissed [13].

A recent systematic review estimated a 7% detection rate of SARS-CoV-2 RNA in semen, independent of age, severity or comorbidities. Interestingly, timing of semen analysis post-COVID-19 diagnosis significantly influences detection rates, with higher prevalence within 11 days post-diagnosis [13].

INTRODUCTION THE GENERAL PROJECT

While a considerable number of scientific papers have been published on the COVID-19 topic, only a few have specifically addressed the effects of SARS-CoV-2 infection on male fertility. Many of these studies encountered limitations, such as small sample sizes, the lack of paired pre- and post-infection samples, absence of confirmed SARS-CoV-2 infection diagnosis by PCR and focusing solely on short-term effects. To address these gaps, Fundació de Recerca Clínic Barcelona – IDIBAPS proposed a multicentric research project aimed at investigating not only the acute effects but also the long-term consequences of SARS-CoV-2 infection from various perspectives.

The project was funded by La Marató de TV3 and includes 34 researchers and clinicians from five main hospitals and research centers of Barcelona (Hospital Clínic Barcelona, Fundació Puigvert-Hospital de Sant Pau, Hospital de la Vall d'Hebrón, SCIAS-Hospital de Barcelona and Universitat Autònoma de Barcelona). This study has been distributed in two phases: the first one, which has already concluded, would compare the semen quality before and after the viral infection (*Retrospective Study*) (**Figure 6**) [32]. The second phase, which my final degree project is based on, focuses on the monitoring of COVID-19 patients periodically, up to six months after infection (*Prospective Study*).

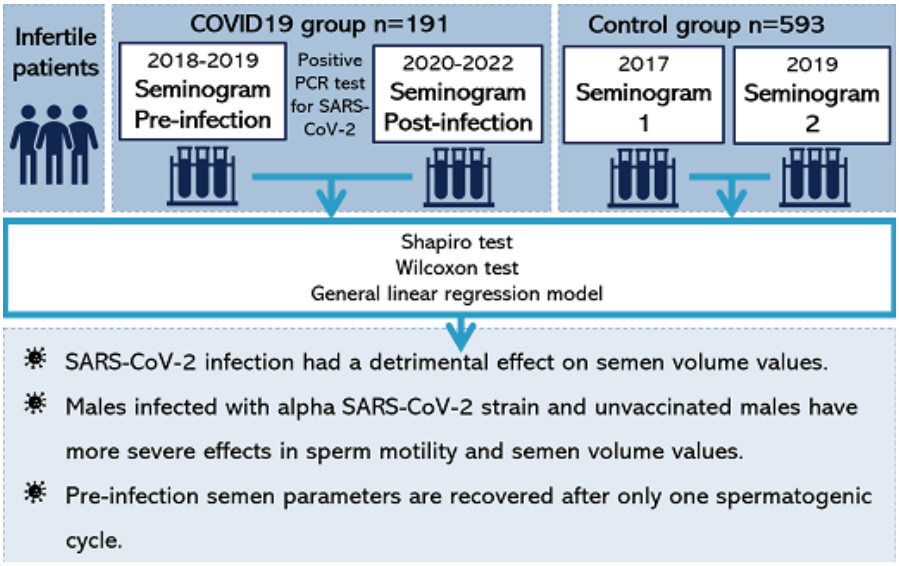


Figure 6. Overview and results from the Retrospective Study. The results obtained are consistent with those reported by other researchers. *Extracted from Jodar et al., 2024* [32]

PROSPECTIVE MULTICENTER STUDY

The strengths of the Retrospective Study were the analysis of paired semen data before and after SARS-CoV-2 infection in a substantial number of patients with a valid positive test for infection and an uninfected control group who underwent two basic semen analyses at different time points. However, as a limitation, most of the patients assessed in the Retrospective Study did not show severe symptoms of COVID-19 disease. In the Prospective Study, patients with severe symptoms of COVID-19 were included and other parameters such as sperm DNA fragmentation, inflammation and hormonal levels were analysed.

HYPOTHESIS

The inclusion of patients with severe COVID-19 symptoms, alongside the assessment of new parameters, will uncover significant differences in semen quality and the recovery of seminal parameters compared to those with mild symptoms.

OBJECTIVES

1) To evaluate the differences in semen quality, including sperm parameters, sperm DNA fragmentation, hormonal levels, a seminal inflammation marker and the presence of SARS-CoV-2 RNA in patients with severe versus mild COVID-19 symptoms at T0.
2) To assess the recovery of abnormalities detected in sperm parameters, sperm DNA fragmentation, hormonal levels and the seminal inflammation marker at different time points post-infection.

METHODOLOGY

The methodological approach of this study (**Figure 7**) was designed to comprehensively investigate the impact of SARS-CoV-2 infection on male fertility, focusing on both COVID-19 disease severity and recovery phases. To achieve this, we employed a diverse array of techniques aimed at examining a broad spectrum of variables and parameters. This rigorous and holistic methodology enabled us to capture the intricate relationships between various biological markers and the progression of the disease, thereby enhancing the complexity and reliability of our findings.

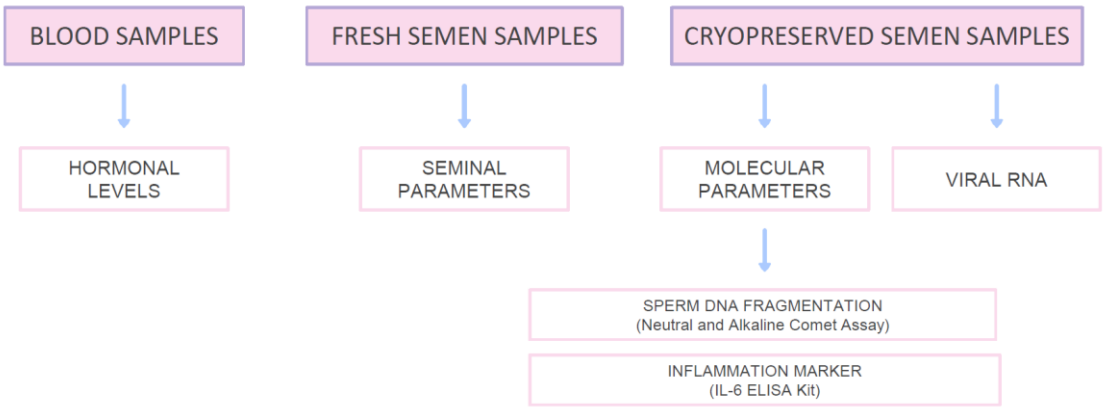


Figure 7. Visual representation of the methodology followed in the Prospective Study.

SAMPLE COLLECTION AND PROCESSING

BLOOD SAMPLES

The Hospital's Assisted Reproduction Unit routinely collects blood samples from infertile patients to analyse key parameters, such as hormonal levels related to male reproductive function, including luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone and inhibin B (INHB). With prior consent, these data were provided to our research group for inclusion in this study.

SEMEN SAMPLES

Samples of leftover semen from men who had visited the Assisted Reproduction Unit at the Clínic Barcelona Hospital and had previously signed an informed consent form were collected. More samples were gathered from other assisted reproduction centers in Barcelona participating in the coordinated project. All of them belonged to patients with a validated infection for SARS-CoV-2 and were categorized based on the severity of symptoms (mild and severe) and the months post-infection (T0*, T3, and T6).

From all samples, an aliquot was saved for Comet assay, a technique where contamination presence is easily discernible under a microscope, thus the samples were used without undergoing any prior purification procedure. Following the assay, samples with higher sperm concentrations underwent purification to ensure contaminant-free specimens for subsequent critical protein studies (not included in this work). This purification process involved a 50% density gradient following thawing. Whether a gradient was employed or not, spermatozoa were consistently separated from seminal plasma in all samples and cryopreserved using the *slow freezing method* - initially in nitrogen vapours and then in liquid nitrogen. All samples utilized test yolk buffer (TYB) as a cryoprotectant, which is the standard used in the clinic.

*T0: less than two weeks after a positive test for SARS-CoV-2.

50% Density gradient

Protocol for sperm sample decontamination using centrifugation density gradient:

1. Centrifuge the sample at 500g for 10 minutes and carefully discard the supernatant.
2. In the meantime, prepare the 50% gradient: mix 1,5 mL of PureSperm® 100 with 1,5 mL of PureSperm® Wash to make a total volume of 3 mL.
3. Resuspend the pellet from Step 1 in PureSperm® Wash. Use an equivalent number of mL as the number of 50% gradients you are preparing. Each gradient receives 1 mL, which corresponds to approximately 60 million spermatozoa (Mz) (one sample can be divided into several gradients).
4. Gently and slowly layer the resuspended sample onto the 50% gradient. Ensure there are no air bubbles between the gradient and the sample.
5. Centrifuge at 300g for 20 minutes with a slow acceleration and deceleration ramp.
6. Discard the supernatant using a circular motion to ensure all debris and round cells are removed. Take care not to disturb the pellet, as it is very unstable.
7. Resuspend the pellet in 1 mL of PureSperm® Wash in a clean tube, similar to Step 3.
8. Use a Makler Counting Chamber® and a bright-field microscope to determine the postgradient concentration.

This system effectively separates normal sperm cells from lymphocytes, epithelial cells, round germinal cells, cellular debris, bacteria and seminal fluid.

Cryopreservation with TYB: slow freezing protocol

Measure the required volume of cryoprotectant and place it in a tube.

1. Slowly add an equal volume of pre-warmed cryoprotectant to the sample (1:1 ratio), adding it drop-by-drop while gently vortexing to ensure thorough mixing.
2. Allow the sample mixed with the cryoprotectant to sit for 10 minutes at room temperature.

3. After incubation, place the tubes at 4ºC for 30 minutes, then expose them to nitrogen vapours at high altitude for 20 minutes, followed by 20 minutes in nitrogen vapours at low altitude.
4. Finally, immerse the tubes in liquid nitrogen for long-term storage.

HORMONAL PARAMETERS IN BLOOD SAMPLES

The levels of testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and inhibin B (INHB) were assessed due to their significant role in spermatogenesis. They are essential components of the male hormonal profile, used to detect potential hormonal imbalances that could lead to fertility issues in men.

SEMINAL PARAMETERS IN FRESH SEMEN SAMPLES

A routine semen analysis was conducted on all samples in accordance with WHO guidelines [2]. The analysis evaluated sperm concentration, seminal volume, total sperm count and contamination by leukocytes and other non-sperm cells. The assessment was performed using a Makler Reusable Sperm Counting Chamber® and CASA software under the microscope.

MOTILITY AND CONCENTRATION

Sperm motility is a crucial functional measure of sperm health, influenced by disturbances in testicular spermatogenesis or effects on the epididymis. This characteristic is classified into four types based on progressivity, form of movement and speed: type A (fast progressive), type B (slow progressive), type C (non-progressive) and type D (immotile) (**Figure 8**) [34].

Spermatozoa can also be classified based on their movement pattern and velocity into three groups: progressive motile (PM), non-progressive (NP) and immotile (**Figure 9**) [34,35]. Progressive motility, which includes both type A and B, refers to sperm that swim in a mostly straight line or in large circles. It is an essential functional trait that enables them to penetrate and migrate through cervical mucus and the oocyte's outer layers, ultimately allowing them to fertilize the oocyte [36].

According to the World Health Organization, the percentage of PM should be greater than 32% and the combined percentage of PM and NP (total motility) should exceed 40% [2]. Samples with values below these thresholds are classified as “asthenozoospermic”.

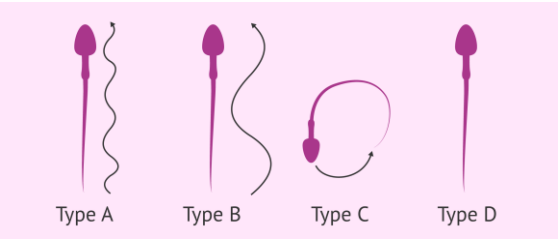


Figure 8. Types of sperm motility that have been described. *Extracted from Ferrando et al., 2023* [34]

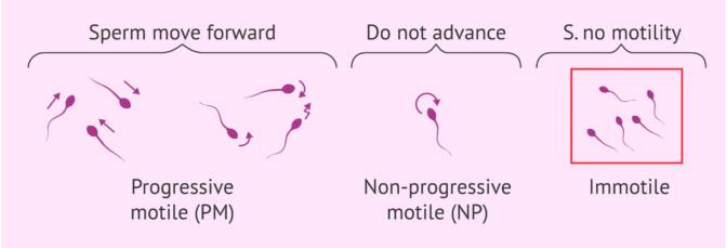


Figure 9. Classification of sperm based on motility. *Extracted from Ferrando et al., 2023* [34]

Both motility and sperm concentration were assessed by the Hospital's Assisted Reproduction Unit by using a Computer-Aided Sperm Analysis (CASA) system with phase contrast microscope optics which enables the performance of reliable assessments of sperm movement pattern characteristics ("kinematics") and sperm concentration in semen [36].

CASA system

A fresh sample of spermatozoa is placed on CASA slides, which contain cell chambers, and kept at 35°C to prevent motility loss due to cold shock. Sperm motility (types A, B and C) and concentration are then assessed through real-time video capture, with the data analysed using CASA software [37].

MORPHOLOGY

The Diff-Quik® staining kit was used to analyse the sperm morphology of the samples. By briefly and consecutively immersing the air-dried smears in the specified solutions, fixation and staining are achieved in just 15 seconds (**Figure 10**) [38]. This staining process enables the quantitative evaluation of both normal and abnormal sperm forms in an ejaculate (**Figure 11**).

Reagents used [39]:

- Fixative Solution: methanol-based solution used to stabilize cellular components.
- Stain Solution I: buffered solution of Eosin Y (anionic dye) which stains basic structures as membranes.
- Stain Solution II: buffered solution of thiazine dyes (cationic dyes) consisting of methylene blue and Azure A. It stains acidic structures as DNA.

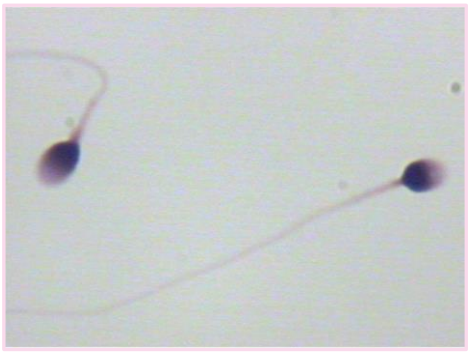


Figure 10. Sperm images for Diff-Quik staining. The head with its acrosome, the midpiece and the tail can be clearly distinguished. *Adapted from Xu et al., 2022* [40]

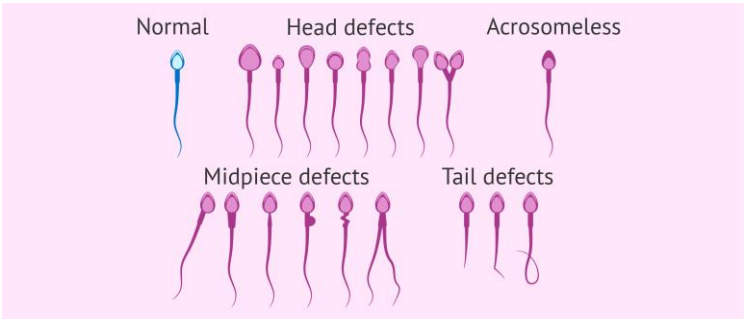


Figure 11. Comparison between normal sperm and different morphological abnormalities that can be found. *Extracted from Ferrando et al., 2023* [34]

MOLECULAR PARAMETERS IN CRYOPRESERVED SEMEN SAMPLES

SPERM DNA FRAGMENTATION (Comet Assay)

The Comet Assay is recognized as the most robust and sensitive single-cell method for detecting DNA breaks, allowing for the quantification of DNA damage in eukaryotic cells. This gel electrophoresis-based technique relies on the principle that DNA damage results in the migration of fragmented DNA towards the anode during electrophoresis, producing a comet-like tail shape under microscopy. By adjusting the pH of the buffer, both single-strand breaks (alkaline comet*) and double-strand breaks (neutral comet) can be evaluated.

Using this method, we assessed the level of sperm DNA fragmentation in samples from patients who had experienced SARS-CoV-2 infection. This enabled us to analyse the pattern of DNA damage in relation to the severity of the infection and the time elapsed since its onset.

Sperm cells are unique in their structure and function, with several adaptations that distinguish them from other cell types. Due to the presence of more complex membrane systems (**Figure 12**) together with a higher condensation of the DNA (**Figure 13**), the elongation and strengthening of the lysis step was required compared to standardized Comet Assay protocols for somatic cells.

*The alkaline comet assay is currently the only technique that allows for the assessment of single-strand DNA breaks.

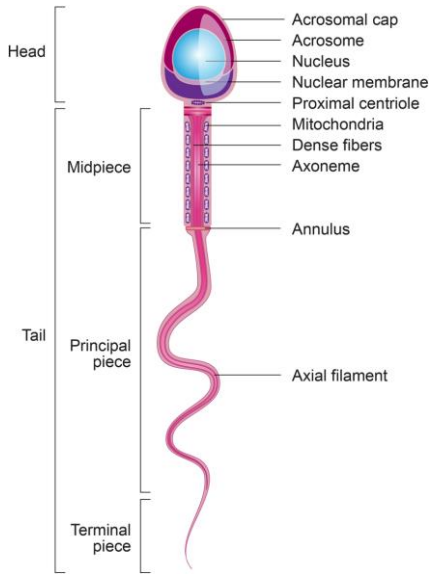


Figure 12. Structural sperm features. Spermatozoa are composed of two main parts: head and tail (or flagellum). The sperm head is constituted basically by the acrosome and nucleus. The sperm tail includes: the neck, the midpiece, the principal piece and terminal piece. *Extracted from Alves et al., 2020* [**41**]

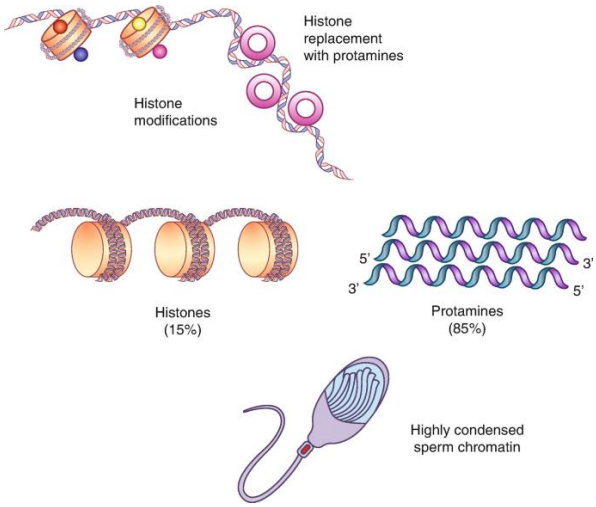


Figure 13. During chromatin condensation, various events proceed in a chronological order. Among these events, histone -protamine replacement is the most critical step. As most of the histones in the chromatin are replaced by protamines, chromatin condenses to a higher order. *Extracted from Okada, 2022 and Marchiani et al., 2020* [**42,43**]

The protocol was adapted from the methodologies of Simon L. and Carrell D. [44] and Ribas-Maynou, J., et al. [45]. The process begins with the preparation of slides pre-coated with normal melting point agarose (NMA), onto which the low melting point agarose (LMA) gel will be applied. Pre-coating with a thin layer of NMA prevents the gel from detaching. The LMA gel, containing approximately 10,000 sperm cells from cryopreserved samples, remains liquid at 37°C, minimizing the risk of cell damage compared to the higher temperatures required by NMA. Two slides are prepared for each sample: one for the alkaline assay and one for the neutral assay.

After preparing the slides with the gels, a three-phase lysis treatment is performed. In the first phase, the slides are submerged for 60 minutes in a solution containing 2.47 M NaCl, 98.9 mM EDTA, 9.8 mM Tris and 1% Triton X-100. In the second phase, 0.5 mM DTT is added to the solution and the slides are submerged for 30 minutes. The final phase involves submerging the slides in the solution now containing 0.2 mM LIS for 90 minutes.

Prior to electrophoresis, all slides are immersed in the appropriate electrophoresis buffer for 30 minutes. The voltage is set to 22V for both the neutral and alkaline assays, with the current controlled (<280 mA) for 10-15 minutes to prevent overheating of the gels. To differentiate between single- and double-strand DNA breaks, electrophoresis is carried out in buffers with either an alkaline pH (0.2M NaOH, 0.001M EDTA, pH 13) or a neutral pH (0.05M Tris Base, 0.15M Sodium Acetate Trihydrate, pH 9) (Figure 14).

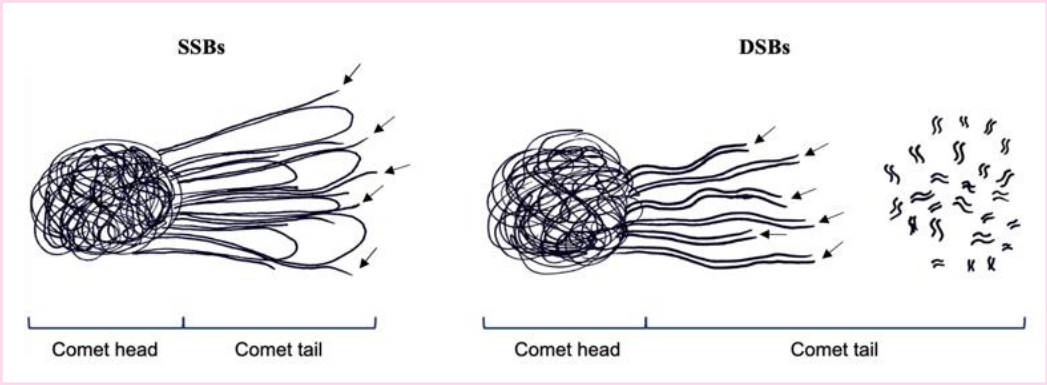


Figure 14. Schematic representation of Single Strand Breaks (SSBs) and Double Strand Breaks (DSBs) of sperm's DNA in Comet Assay.

After electrophoresis, the slides are gently rinsed with purified water using the Milli-Q system and then fixed with 70% ethanol. To conclude the process, the gels are stained with SYBR® Gold, a fluorescent dye specific for DNA and cells are analysed for fragmentation under a fluorescent microscope. Fragmented nuclei display a comet-like tail, while non-fragmented nuclei retain a large, round shape (Figure 15). The final result is expressed as the percentage of fragmented cells relative to the total number of cells counted, typically ranging from 60 to 120 cells per sample.

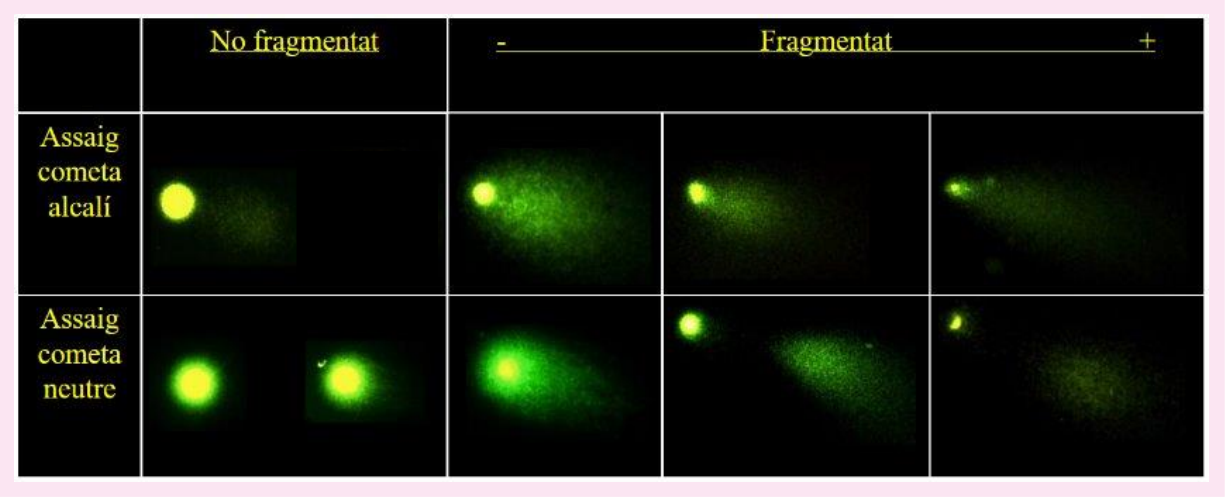


Figure 15. Summary diagram of the different sperm nuclei that can be found as a result of a neutral and alkaline comet assay, classified from least to greatest sperm DNA fragmentation.

INFLAMMATION ASSESSED IN SEMINAL FLUID

Proinflammatory cytokines are signalling molecules released by both immune cells and tissue cells in response to external triggers and other cytokines. These molecules are key players in initiating and regulating inflammatory reactions and are involved in various biological processes. In seminal plasma, cytokines are naturally present, originating from various components of the male reproductive system, such as Leydig cells, Sertoli cells, the epididymis and the prostate [46]. For instance, IL-6 is involved in processes such as spermatogonial proliferation, germ cell differentiation, Sertoli cell steroidogenesis and protein secretion.

It is well-known that cytokine levels, including IL-6, IL-8 and tumour necrosis factor α (TNF-α), rise in response to pathological conditions or when sperm parameters are disrupted [46]. Given this, stored seminal plasma samples were used to evaluate inflammation by measuring IL-6 levels in a selected subgroup of patients with SARS-CoV-2 infection at both T0 and T3. Previous hospital tests had confirmed the presence of detectable IL-6 levels in seminal plasma. To measure this parameter, an enzyme-linked immunosorbent assay (ELISA) kit specific to IL-6 was employed at the hospital.

PRESENCE OF SARS-CoV-2 RNA IN SEMEN

In accordance with standard biosafety protocols from the Microbiology Department at Hospital Clínic, cryopreserved T0 semen samples were processed using density gradient centrifugation to separate the seminal plasma from the cellular fraction. Both fractions were then tested for the presence of SARS-CoV-2 RNA using reverse transcription polymerase chain reaction (RT-PCR), targeting the viral E protein gene.

STATISTICAL ANALYSIS

The statistical analysis was conducted in Jamovi® [47]. The Shapiro-Wilk test was applied to evaluate the normality of the studied variables. For comparing results based on severity, an independent samples test was selected, specifically the Mann-Whitney U test. Conversely, the Wilcoxon W test, a nonparametric test designed for paired samples, was used for comparing results across different time points. Both tests needed to be nonparametric due to the distribution of our samples (non-normal).

RESULTS AND DISCUSSION

DESCRIPTIVE STATISTICS AND NORMALITY

The first step of the statistical analysis involved creating a table that summarized the descriptive statistics of our variables and assessing their distribution (normal or non-normal) (Table 3). The Shapiro-Wilk test was employed to evaluate normality, given the relatively small sample size (N ≈ 50). The results indicated that only two parameters (Volume and INHB) followed a normal distribution. Consequently, all variables were treated as non-normal for subsequent analyses.

Guide for interpreting the Shapiro-Wilk test:

- P > 0,05: the null hypothesis is not rejected (normal distribution)
- P < 0,05: the null hypothesis is rejected (non-normal distribution)

Based on the p-values obtained from this test, we concluded that it was appropriate to include the median and interquartile range (IQR) in the descriptive table, alongside the mean and standard deviation. The median and IQR are more statistically accurate for representing the spread of values in samples that do not follow a normal distribution, as they are less affected by extreme values or outliers.

Reviewing the results from this table, we observed that several parameters—progressive motility, single-strand DNA fragmentation, IL-6 and FSH—fell outside the established normal thresholds. A key consideration in drawing conclusions is the significant variability within these samples, which complicates forming a general interpretation. Despite this, it seems that patients with SARS-CoV-2 infection experience alterations in one or more parameters related to male reproductive health.

Table 3. Descriptive summary of the analysed variables. The normal threshold values are based on WHO indications (*) [2], previous results of our research group (#) and published literature (~) [46]. From the studied samples (N = 58): 24 patients were oligospermic (low sperm count), 31 patients were asthenozoospermic (poor sperm motility) and 3 patients were normozoospermic (normal values). SD: Standard Deviation; IQR: Interquartile Range; W: Shapiro-Wilk statistical value

									Shapiro-Wilk	
	N	Mean	SD	Median	IQR	Minimum	Maximum	Normal thresholds	W	P-value
Seminal volume (mL)	58	2.60	1.27	2.50	1.85	0.20	5.70	> 1.4*	0.980	0.467
Sperm concentration (Mz/mL)	58	57.25	63.18	30.03	88.31	0	270.23	> 16*	0.833	<.001
Total sperm (Mz)	58	152.19	240.51	54.47	173.15	0	1540.31	> 39*	0.610	<.001
Motility type A (%)	58	13.40	12.98	11.93	14.90	0	64.04	-	0.856	<.001
Motility type B (%)	58	17.59	12.95	16.07	15.54	0	60	-	0.913	<.001
Progressive motility (A+B) (%)	58	30.99	21.66	27.32	25.12	0	82.55	> 30*	0.938	0.005
Normal forms (%)	50	6.48	6.30	5	7.75	0	25	> 4*	0.849	<.001
Single-strand DNA fragmentation (%)	38	32.66	20.43	27.84	22.01	6.89	100	20.06-33.12 (medium) > 33.12 (high)#	0.833	<.001
Double-strand DNA fragmentation (%)	37	24	18.49	18.79	15.81	4.76	98.54	27.72-36.69 (medium) > 36.69 (high)#	0.779	<.001
IL6 seminal plasma levels (pg/mL)	18	33.62	44.92	25.05	20.10	2	198.60	< 21.5~	0.608	<.001
LH blood levels (U/L)	50	4.41	2.05	4.10	2.93	1.70	9.89	1.5-7.5*	0.927	0.004
FSH (U/L)	50	8.61	9.79	6.18	4.50	1.06	60.63	1.7-8*	0.554	<.001
INHB (ng/L)	46	164.21	71.86	163.90	104.85	52.30	346.90	120-400*	0.970	0.268
Testosterone (ng/dL)	50	450.59	262.26	424.90	236.75	4.11	1123	275-850*	0.948	0.029

OBJECTIVE 1: Comparison of seminal parameters, sperm DNA fragmentation, hormonal levels, inflammation and presence of SARS-CoV-2 RNA in patients classified according to the severity level (mild or severe) of COVID-19 disease at T0.

The goal of this part of the statistical analysis was to compare the results obtained for the studied parameters between the two severity groups using the T0 samples. Since these samples were not "paired" (they did not come from the same patients), we employed the Mann-Whitney U test, which is the non-parametric version of the classical Student's t-test. (**Annex 1**)

SEMINAL PARAMETERS

Surprisingly, semen volume, which was expected to be significantly reduced based on the Retrospective Study [32], did not show a marked decrease. This led us to hypothesize that while there may be a significant decrease in semen volume between pre- and post-infection data, the severity of the infection itself might not play a significant role in this reduction once the infection is present. This finding contrasts with other seminal parameters, which were more directly affected by the severity of the disease.

The decrease in sperm concentration ($P = 0.022$) (**Figure 16A**) and total sperm count ($P = 0.002$), which are closely related, were statistically significant. Additionally, both motility A ($P < 0.001$) and motility B ($P = 0.021$), and consequently progressive motility (A + B) ($P = 0.002$) (**Figure 16B**), were notably affected by the severity of the SARS-CoV-2 infection. The percentage of sperm with motility A, which represents fast-progressive sperm that swim quickly and in a straight

direction, showed the most severe decline, suggesting that these sperm may have become immotile or even died.

The potential mechanisms behind these findings could involve the impact of systemic inflammation, oxidative stress or direct viral effects on the reproductive organs, which may vary based on the severity of the infection. However, the relatively stable semen volume might indicate that some seminal parameters are more resilient to the effects of severe infection or that other factors, such as pre-existing conditions or individual patient responses, play a role.

In our study, patients were divided into two severity subgroups based on the severity of their symptoms and whether they required hospitalization. However, we did not focus on the values of any specific parameter. A recently published study evaluating the impact of increased body temperature caused by fever during infection demonstrated that patients categorized as having moderate-to-high fever experienced significant reductions in total sperm count, concentration and progressive motility [11]. These findings support our results, as the close relationship between the severity of an infection and fever has been well-established for some time.

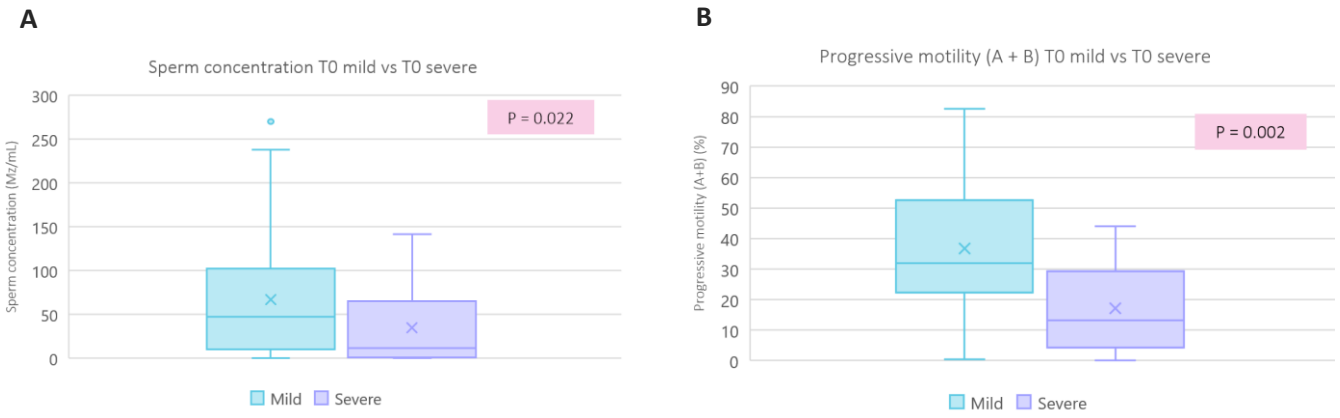


Figure 16A and 16B. Boxplots representing the differences observed in A: sperm concentration (P = 0,022 Mild: 66,56 ± 66,15 Severe: 34,79 ± 50,24) and B: progressive motility (P = 0,002 Mild: 36,75 ± 21,68 Severe: 17,11 ± 14,38) between T0 samples from mild and severe group samples. Mean ± SD

SPERM DNA FRAGMENTATION

Regarding sperm DNA fragmentation, both single-strand (P = 0.016) (**Figure 17A**) and double-strand (P = 0.019) (**Figure 17B**) sperm DNA fragmentation showed a significant increase in the severe group. These findings align with the known pathophysiology of SARS-CoV-2 infection, which triggers cellular oxidative stress, leading to sperm DNA breaks. This oxidative stress can result from an overactive immune response, increased production of reactive oxygen species and inflammation, all of which are characteristic of severe COVID-19 cases.

Previous studies have linked sperm DNA fragmentation to poor embryonic development, reduced implantation rates and higher miscarriage rates [48-50]. This raises important considerations for fertility treatments, as patients recovering from severe COVID-19 may require additional evaluation for sperm DNA fragmentation before undergoing assisted reproduction procedures.

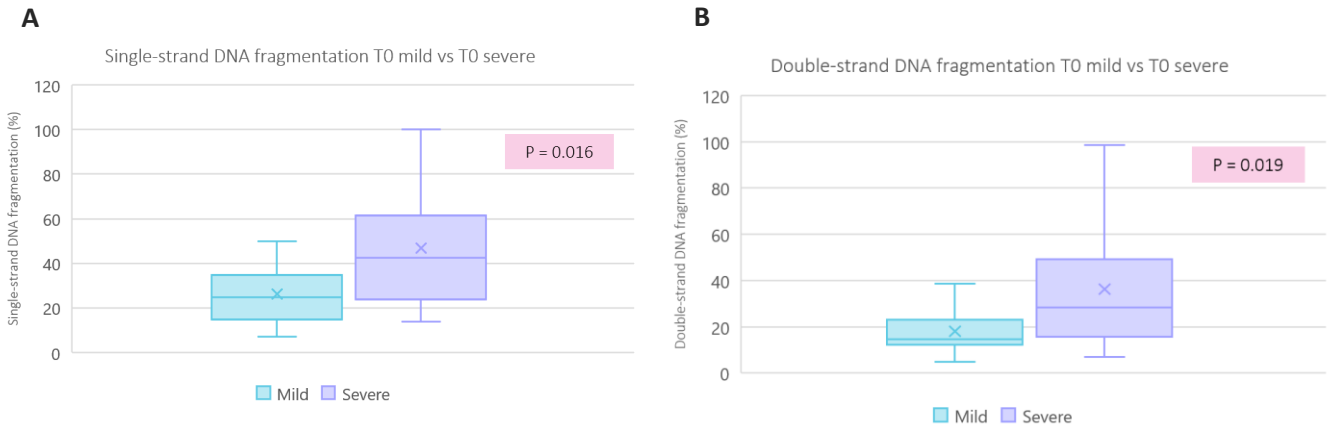


Figure 17A and 17B. Boxplots representing the differences observed in A: single-strand (P = 0,016 Mild: 26,15 ± 11,39 Severe: 46,76 ± 28,14) and B: double-strand (P = 0,019 Mild: 18,10 ± 9,22 Severe: 36,30 ± 26,25) sperm DNA fragmentation between T0 samples from mild and severe group samples. Mean ± SD

HORMONAL LEVELS

Although FSH levels appeared slightly above the normal threshold in both the mild and severe patient groups (Mild: 8.54 ± 7.27 Severe: 8.74 ± 13.70), no significant differences were observed in any hormone levels in this part of the study.

SEMINAL INFLAMMATION MARKER

Regarding inflammation, IL-6 levels were observed to be above normal in both groups (Mild: 38.53 ± 62.26 Severe: 28.71 ± 18.91), although the differences between them were not statistically significant. This may be due to an underlying inflammation present in all these patients, which, while detectable, is not distinct enough to differentiate between severity levels. Another factor to consider is the high variability in the data, particularly in the mild group.

PRESENCE OF SARS-CoV-2 RNA IN SEMEN

No viral RNA was found in the semen of the T0 samples analysed. Although testicular damage caused by SARS-CoV-2 has been observed, whether the presence of the virus can be detected in semen remains under debate. Some studies have reported positive identification of SARS-CoV-2 like particles in a small percentage of patients, while others, like ours, reveal the absence of viral mRNA in semen. Therefore, this topic is still unclear, but increasing evidence suggests that the effects on seminal parameters are likely to be due to infection-related symptoms such

as fever and viral orchitis (inflammation of one or both testicles), rather than direct action of the virus on sperm.

Given these results, the clinical implications are significant. While some of the parameters may not be reliable indicators of infection severity, reductions in sperm concentration and motility and increased levels of sperm DNA fragmentation could have important implications for fertility, especially in severe cases of COVID-19. It's important to note that we did not account for the patients' baseline levels, which could have been affected, considering these patients were most infertile and part of the Assisted Reproduction Unit.

OBJECTIVE 2: Comparison of recovery levels of semen parameters, hormonal levels and inflammation in patients classified according to the severity level (mild or severe) of COVID-19 disease at T0 and time post-infection.

After assessing the impact of severity, we shifted our focus to analysing the recovery of parameters over time. We conducted three separate statistical analyses: one including all samples, one for mild cases and one for severe cases. This was done to determine whether recovery was also influenced by the severity of the infection, as we hypothesized. In this analysis, since the samples were paired, we used the non-parametric Wilcoxon Signed-Rank Test to evaluate the data. (**Annex 2**)

SEMINAL PARAMETERS

Although semen volume appears to decrease from T0 to T3 as in the Retrospective Study [**32**], this change is notably significant only in the mild group when comparing T0 and T6. This finding is consistent with most of studies on this topic, which have reported significant reductions in semen parameters such as sperm count, sperm concentration and total motility, while noting no substantial changes in semen volume.

Interestingly, sperm concentration shows a significant increase in the severe group at three months (P = 0.003) and six months post-infection (P = 0.020) (**Figure 18A**). A similar trend is observed for total sperm counts, with significant increases noted from T0 to T3 (P = 0.030) and from T0 to T6 (P = 0.004). Additionally, sperm motility improves significantly during the post-infection period, particularly in the severe group. Type A motility increases notably from T0 to T3 (P = 0.012) and from T0 to T6 (P = 0.004), while type B motility also shows significant improvement from T0 to T3 (P = 0.011) and from T0 to T6 (P = 0.008). Consequently, progressive motility recovers significantly from T0 to T3 (P = 0.002) and from T0 to T6 (P = 0.004) (**Figure 18B**).

These improvements highlight a positive trend in seminal parameters during post-infection recovery, which is consistent with findings reported in the literature. It appears that seminal parameters experience their most significant decline within the first 30 days following SARS-CoV-2 infection, but then gradually recover. In most studies (including the Retrospective Study performed by the group [32]), by 90 days post-infection, these parameters show no significant difference compared to levels observed before the SARS-CoV-2 infection [14].

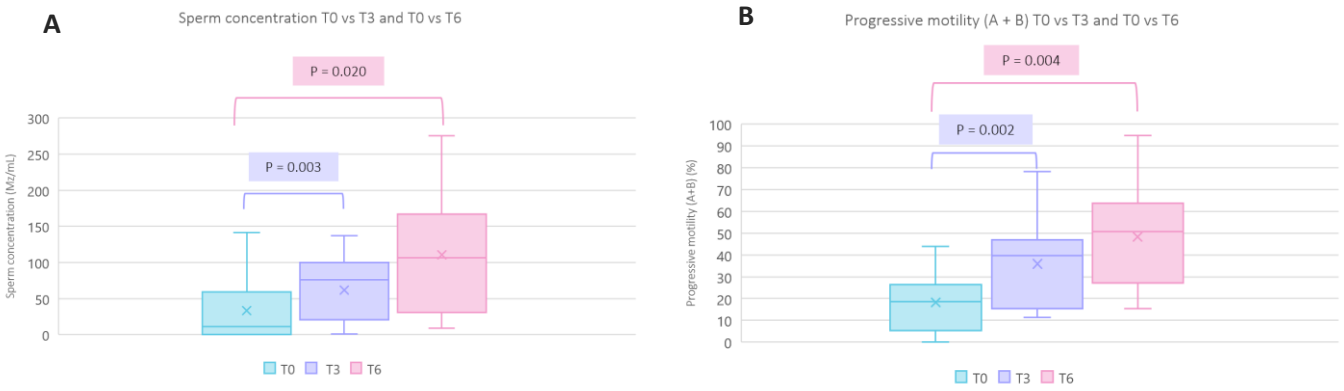


Figure 18A and 18B. Boxplots representing the differences observed in the severe group in A: sperm concentration between T0-T3 (P = 0,003) and T0-T6 (P = 0,020) and B: progressive motility T0-T3 (P = 0,002) and T0-T6 (P = 0,004).

SPERM DNA FRAGMENTATION

In the severe group, there is a noticeable trend of both single-strand and double-strand DNA fragmentation decreasing from T0 to T6. However, this recovery is not statistically significant.

HORMONAL LEVELS

FSH was the only hormone that showed a significant decrease during COVID-19 recovery, particularly in the severe subgroup (**Figure 19**). Although FSH is essential for fertility, an elevated level can indicate impaired spermatogenesis. When the brain detects that the testicles are producing less sperm than normal, it releases more FSH into the bloodstream to stimulate sperm production [51]. Numerous studies have shown that infertile men with reduced testicular volume tend to have significantly higher FSH levels, along with lower sperm count and motility. Therefore, the decrease observed in our study suggests that spermatogenesis is recovering during the post-infection months as we have previously observed with the seminal parameters.

Nevertheless, it would be important to closely examine the two outliers visible in the graph to understand how significantly their reproductive profiles differ and to determine if they could account for the variations observed in FSH levels.

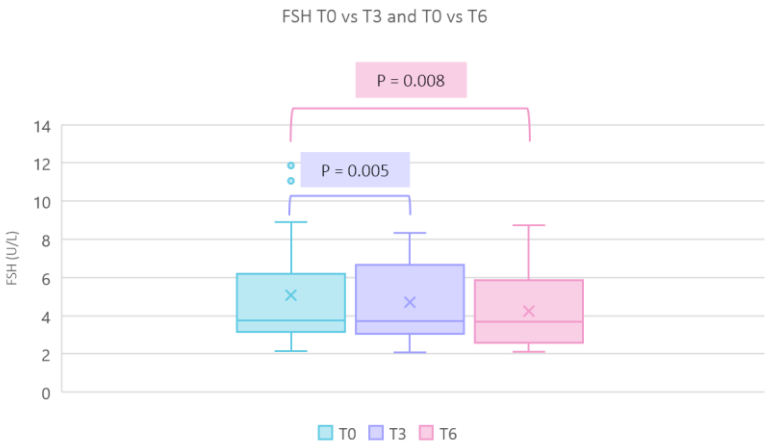


Figure 19. Boxplot representing the differences observed in the severe group in FSH levels between T0-T3 (P = 0,005) and T0-T6 (P = 0,008).

SEMINAL INFLAMMATION MARKER

IL-6 levels did not provide substantial information in this part of the study due to the insufficient number of samples with available data. In future research, it will be important to collect additional samples and measure IL-6 levels at various time points post-infection to draw more definitive conclusions. Additionally, including other inflammatory markers in the analysis could offer a more comprehensive understanding of the inflammatory response over time.

Comparing the most distant time points, T0 and T6, there appears to be an improvement in the parameters affected by SARS-CoV-2 infection, particularly in the severe cases. This improvement is notably evident in seminal parameters, consistent with existing literature.

Normal human spermatogenesis generally takes about three months, during which semen volume, sperm concentration and motility can fluctuate due to physiological variations [13]. This variability, related to baseline levels, should be accounted for and further investigated in future research.

CONCLUSION

Our results indicate that the male reproductive system, and therefore fertility, could be affected by SARS-CoV-2 infection. The severity of the infection seems to play a significant role in seminal parameters, with both sperm count and motility being more significantly reduced in severe cases compared to mild cases. Although the mild group also shows noticeable changes, these are less pronounced. We observed a similar trend with sperm DNA fragmentation, which could be driven by oxidative stress induced by the infection.

When analysing these parameters over time, the results are encouraging. A gradual recovery of the affected values is observed in both mild and severe cases. By three months (the duration of a human spermatogenic cycle), it appears that most patients do not experience lasting fertility issues aligning with the results obtained in the Retrospective Study [32]. This is an important consideration for assisted reproduction processes, as a waiting period of three months may be advisable to minimize potential complications in pregnancy or fetal development. No presence of viral RNA was found in semen, reinforcing the increasingly accepted idea within the scientific community that the effects of the virus on sperm are likely due to indirect consequences of the infection—such as elevated body temperature, inflammation and oxidative stress—rather than the direct presence of the virus in this biological fluid.

These findings underscore the importance of monitoring male reproductive health in patients recovering from COVID-19, particularly in those with severe infections. While the observed recovery in seminal parameters is reassuring, the potential temporary impact on fertility highlights the need for caution, especially in cases involving assisted reproduction. Future studies should investigate potential protective factors and assess whether similar effects are observed with other viral infections. Understanding these dynamics will be crucial in managing public health implications in the context of current and future pandemics.

LIST OF ABBREVIATIONS

ACE2: Angiotensin Converting Enzyme 2

ARDS: Acute Respiratory Distress Syndrome

AXL: AXL Receptor Tyrosine Kinase

COVID-19: Coronavirus disease 2019

CoVs: Coronaviruses

DNA: Deoxyribonucleic acid

DSBs: Double Strand Breaks

ELISA: Enzyme-linked immunosorbent assay

FSH: Follicle-stimulating hormone

IL-6: Interleukin 6

INHB: Inhibin B

IQR: Interquartile range

LH: Luteinizing hormone

LMA: Low melting agarose

MERS: Middle East respiratory syndrome (MERS-CoV)

MP: Progressive motility

Mz: Millions of spermatozoa

NMA: Normal melting agarose

NP: Non-progressive motility

RAS: Renin-Angiotensin System

RBD: Receptor-binding domain

RNA: Ribonucleic acid

RT-PCR: Reverse transcription polymerase chain reaction

SARS: Severe Acute Respiratory Syndrome (SARS-CoV / SARS-CoV 2)

S-protein: Spike protein

SSBs: Single Strand Breaks

TMPRSS2: Transmembrane Serine Protease 2

TYB: Test yolk buffer

WHO: World Health Organization

REFERENCES

1. Vander Borght, M., & Wyns, C. (2018). Fertility and infertility: Definition and epidemiology. *Clinical biochemistry*, *62*, 2–10. https://doi.org/10.1016/j.clinbiochem.2018.03.012

2. World Health Organization. WHO laboratory manual for the Examination and processing of human semen. 2021. https://www.who.int/publications/i/item/9789240030787

3. Garolla, A., Torino, M., Sartini, B., Cosci, I., Patassini, C., Carraro, U., & Foresta, C. (2013). Seminal and molecular evidence that sauna exposure affects human spermatogenesis. *Human reproduction (Oxford, England)*, *28*(4), 877–885. https://doi.org/10.1093/humrep/det020

4. Payne, D. C., Smith-Jeffcoat, S. E., Nowak, G., Chukwuma, U., Geibe, J. R., Hawkins, R. J., Johnson, J. A., Thornburg, N. J., Schiffer, J., Weiner, Z., Bankamp, B., Bowen, M. D., MacNeil, A., Patel, M. R., Deussing, E., CDC COVID-19 Surge Laboratory Group, & Gillingham, B. L. (2020). SARS-CoV-2 Infections and Serologic Responses from a Sample of U.S. Navy Service Members - USS Theodore Roosevelt, April 2020. *MMWR. Morbidity and mortality weekly report*, *69*(23), 714–721. https://doi.org/10.15585/mmwr.mm6923e4

5. Levine, K. S., Leonard, H. L., Blauwendraat, C., Iwaki, H., Johnson, N., Bandres-Ciga, S., Ferrucci, L., Faghri, F., Singleton, A. B., & Nalls, M. A. (2023). Virus exposure and neurodegenerative disease risk across national biobanks. *Neuron*, *111*(7), 1086–1093.e2. https://doi.org/10.1016/j.neuron.2022.12.029

6. Antushevich, H. (2020). Interplays between inflammasomes and viruses, bacteria (pathogenic and probiotic), yeasts and parasites. In *Immunology Letters* (Vol. 228). https://doi.org/10.1016/j.imlet.2020.09.004

7. Gusev, E., Sarapultsev, A., Solomatina, L., & Chereshnev, V. (2022). Sars-Cov-2-Specific Immune Response and the Pathogenesis of COVID-19. In *International Journal of Molecular Sciences* (Vol. 23, Issue 3). https://doi.org/10.3390/ijms23031716

8. Mahmood, Z., Alrefai, H., Hetta, H. F., Kader, H. A., Munawar, N., Rahman, S. A., Elshaer, S., Batiha, G. E. S., & Muhammad, K. (2020). Investigating virological, immunological, and pathological avenues to identify potential targets for developing covid-19 treatment and prevention strategies. In *Vaccines* (Vol. 8, Issue 3). https://doi.org/10.3390/vaccines8030443

9. Team, B. (2020). Mechanism of SARS-CoV-2 Viral Entry. https://app.biorender.com/biorender-templates/figures/all/t-5f99eb1307ef9a009f1c61e0-mechanism-of-sars-cov-2-viral-entry (Access: July 13)

10. Harb, J., Debs, N., Rima, M., Wu, Y., Cao, Z., Kovacic, H., Fajloun, Z., & Sabatier, J. M. (2022). SARS-CoV-2, COVID-19, and Reproduction: Effects on Fertility, Pregnancy, and Neonatal Life. In *Biomedicines* (Vol. 10, Issue 8). https://doi.org/10.3390/biomedicines10081775

11. Zhang, G., Zhi, W., Ye, F., Xiong, D., Zhang, Y., Liu, F., Zhao, Y., Du, X., Wu, Y., Hou, M., Liu, J., Wei, J., Silang, Y., Xu, W., Zeng, J., Chen, S., & Liu, W. (2024). Systematic analyses of the factors influencing sperm quality in patients with SARS-CoV-2 infection. *Nature (Scientific Reports)*, *14*(1). https://doi.org/10.1038/s41598-024-58797-y

12. *Human Protein Atlas*. (n.d.). https://www.proteinatlas.org (Access: July 25)

13. Ata, B., Vermeulen, N., Mocanu, E., Gianaroli, L., Lundin, K., Rautakallio-Hokkanen, S., Tapanainen, J. S., & Veiga, A. (2023). SARS-CoV-2, fertility and assisted reproduction. In *Human Reproduction Update* (Vol. 29, Issue 2). https://doi.org/10.1093/humupd/dmac037

14. Zhang, G., Zhi, W., Ye, F., Xiong, D., Zhang, Y., Liu, F., Zhao, Y., Du, X., Wu, Y., Hou, M., Liu, J., Wei, J., Silang, Y., Xu, W., Zeng, J., Chen, S., & Liu, W. (2024). Systematic analyses of the factors influencing sperm quality in patients with SARS-CoV-2 infection. *Nature (Scientific Reports)*, *14*(1). https://doi.org/10.1038/s41598-024-58797-y

15. SeyedAlinaghi, S., Mirzapour, P., Dadras, O., Pashaei, Z., Karimi, A., MohsseniPour, M., Soleymanzadeh, M., Barzegary, A., Afsahi, A. M., Vahedi, F., Shamsabadi, A., Behnezhad, F., Saeidi, S., Mehraeen, E., & Shayesteh Jahanfar (2021). Characterization of SARS-CoV-2 different variants and related morbidity and mortality: a systematic review. *European journal of medical research*, *26*(1), 51. https://doi.org/10.1186/s40001-021-00524-8

16. Clausen, T. M., Sandoval, D. R., Spliid, C. B., Pihl, J., Perrett, H. R., Painter, C. D., Narayanan, A., Majowicz, S. A., Kwong, E. M., McVicar, R. N., Thacker, B. E., Glass, C. A., Yang, Z., Torres, J. L., Golden, G. J., Bartels, P. L., Porell, R. N., Garretson, A. F., Laubach, L., Feldman, J., … Esko, J. D. (2020). SARS-CoV-2 Infection Depends on Cellular Heparan Sulfate and ACE2. *Cell*, *183*(4), 1043–1057.e15. https://doi.org/10.1016/j.cell.2020.09.033

17. Mayi, B. S., Leibowitz, J. A., Woods, A. T., Ammon, K. A., Liu, A. E., & Raja, A. (2021). The role of Neuropilin-1 in COVID-19. *PLoS pathogens*, *17*(1), e1009153. https://doi.org/10.1371/journal.ppat.1009153

18. Hopkins, C., Lechien, J. R., & Saussez, S. (2021). More that ACE2? NRP1 may play a central role in the underlying pathophysiological mechanism of olfactory dysfunction in COVID-19 and its association with enhanced survival. *Medical hypotheses*, *146*, 110406. https://doi.org/10.1016/j.mehy.2020.110406

19. Kyrou, I., Randeva, H. S., Spandidos, D. A., & Karteris, E. (2021). Not only ACE2-the quest for additional host cell mediators of SARS-CoV-2 infection: Neuropilin-1 (NRP1) as a novel SARS-CoV-2 host cell entry mediator implicated in COVID-19. *Signal transduction and targeted therapy*, *6*(1), 21. https://doi.org/10.1038/s41392-020-00460-9

20. Wang, S., Qiu, Z., Hou, Y., Deng, X., Xu, W., Zheng, T., Wu, P., Xie, S., Bian, W., Zhang, C., Sun, Z., Liu, K., Shan, C., Lin, A., Jiang, S., Xie, Y., Zhou, Q., Lu, L., Huang, J., & Li, X. (2021). AXL is a candidate receptor for SARS-CoV-2 that promotes infection of pulmonary and bronchial epithelial cells. *Cell research*, *31*(2), 126–140. https://doi.org/10.1038/s41422-020-00460-y

21. Wang, K., Chen, W., Zhang, Z., Deng, Y., Lian, J. Q., Du, P., Wei, D., Zhang, Y., Sun, X. X., Gong, L., Yang, X., He, L., Zhang, L., Yang, Z., Geng, J. J., Chen, R., Zhang, H., Wang, B., Zhu, Y. M., Nan, G., … Chen, Z. N. (2020). CD147-spike protein is a novel route for SARS-CoV-2 infection to host cells. *Signal transduction and targeted therapy*, *5*(1), 283. https://doi.org/10.1038/s41392-020-00426-x

22. Fenizia, C., Galbiati, S., Vanetti, C., Vago, R., Clerici, M., Tacchetti, C., & Daniele, T. (2021). SARS-CoV-2 Entry: At the Crossroads of CD147 and ACE2. *Cells*, *10*(6), 1434. https://doi.org/10.3390/cells10061434

23. Ibrahim, I. M., Abdelmalek, D. H., & Elfiky, A. A. (2019). GRP78: A cell's response to stress. *Life sciences*, *226*, 156–163. https://doi.org/10.1016/j.lfs.2019.04.022

24. Elfiky, A. A., Ibrahim, I. M., Amin, F. G., Ismail, A. M., & Elshemey, W. M. (2021). COVID-19 and Cell Stress. *Advances in experimental medicine and biology*, *1318*, 169–178. https://doi.org/10.1007/978-3-030-63761-3_10

25. Shahriari Felordi, M., Memarnejadian, A., Najimi, M., & Vosough, M. (2021). Is There any Alternative Receptor for SARS-CoV-2?. *Cell journal*, *23*(2), 247–250. https://doi.org/10.22074/cellj.2021.7977

26. Gonzalez-Gronow, M., Gopal, U., Austin, R. C., & Pizzo, S. V. (2021). Glucose-regulated protein (GRP78) is an important cell surface receptor for viral invasion, cancers, and neurological disorders. *IUBMB life*, *73*(6), 843–854. https://doi.org/10.1002/iub.2502

27. Carlos, A. J., Ha, D. P., Yeh, D. W., Van Krieken, R., Tseng, C. C., Zhang, P., Gill, P., Machida, K., & Lee, A. S. (2021). The chaperone GRP78 is a host auxiliary factor for SARS-CoV-2 and GRP78 depleting antibody blocks viral entry and infection. *The Journal of biological chemistry*, *296*, 100759. https://doi.org/10.1016/j.jbc.2021.100759

28. Gusev, E., Sarapultsev, A., Hu, D., & Chereshnev, V. (2021). Problems of pathogenesis and pathogenetic therapy of COVID-19 from the perspective of the general theory of pathological systems (General pathological processes). In *International Journal of Molecular Sciences* (Vol. 22, Issue 14). https://doi.org/10.3390/ijms22147582

29. Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S., & Dhama, K. (2014). Oxidative stress, prooxidants, and antioxidants: the interplay. *BioMed research international*, *2014*, 761264. https://doi.org/10.1155/2014/761264

30. Ambar, R. F., Parekh, N., & Agarwal, A. (2020). Recent advances and controversies in diagnosing and treating male infertility. *Faculty reviews*, *9*, 22. https://doi.org/10.12703/r/22

31. Aitken, R. J., & Baker, M. A. (2004). Oxidative stress and male reproductive biology. *Reproduction, fertility, and development*, *16*(5), 581–588. https://doi.org/10.10371/RD03089

32. Jodar, M., Barral, Y., Leiva, M., Castillo, J et al. (2024). Reversible effects of SARS-CoV-2 on semen parameters. In *Systems Biology in Reproductive Medicine* [Manuscript accepted for publication].

33. *Global Health 50/50*. (n.d.). https://globalhealth5050.org (Access: July 30)

34. Ferrando Gilabert, N., Aura Masip, M.., Ortega López, L., Reus, R., & Velasco Álvarez, M. (2023, July 3). *What values are normal in sperm motility analysis?* inviTRA. https://www.invitra.com/en/analysis-of-sperm-motility/ (Access: July 19)

35. Dcunha, R., Hussein, R. S., Ananda, H., Kumari, S., Adiga, S. K., Kannan, N., Zhao, Y., & Kalthur, G. (2022). Current Insights and Latest Updates in Sperm Motility and Associated Applications in Assisted Reproduction. *Reproductive sciences (Thousand Oaks, Calif.)*, *29*(1), 7–25. https://doi.org/10.1007/s43032-020-00408-y

36. Mortimer, D., & Mortimer, S. T. (2013). Computer-Aided Sperm Analysis (CASA) of sperm motility and hyperactivation. *Methods in molecular biology (Clifton, N.J.)*, *927*, 77–87. https://doi.org/10.1007/978-1-62703-038-0_8

37. *Motility (CASA) | Breed Diagnostics*. (n.d.). https://www.breeddiagnostics.com.au/individual-assessment-pages/motility-computer-assisted-sperm-assessment-casa (Access: July 19)

38. Agarwal, A., Gupta, S., Sharma, R. (2016). Sperm Morphology Stain (Diff-Quik®). In: Agarwal, A., Gupta, S., Sharma, R. (eds) Andrological Evaluation of Male Infertility. Springer, Cham. https://doi.org/10.1007/978-3-319-26797-5_9

39. Microptic. (2024, July 2). *Diff-Quik™ stain for sperm morphology analysis - HT CASA*. HT CASA. https://www.micropticsl.com/products/disposables/morphology/diff-quik/#:~:text=The%20Diff%2DQuik%E2%84%A2%20staining,stained%20in%20only%2015%20seconds (Access: July 25)

40. Xu, Y. H., Lu, J. C., & Tang, S. S. (2022). Effects of six kinds of sperm staining methods on human sperm size and evaluation of their staining effects. *Journal of Clinical Laboratory Analysis*, *36*(12). https://doi.org/10.1002/jcla.24794

41. Alves, Maíra & Carvalho Celeghini, Eneiva Carla & Belleannée, Clémence. (2020). From Sperm Motility to Sperm-Borne microRNA Signatures: New Approaches to Predict Male Fertility Potential. Frontiers in Cell and Developmental Biology. 8. 791. https://doi.org/10.3389/fcell.2020.00791

42. Okada Y. (2022). Sperm chromatin condensation: epigenetic mechanisms to compact the genome and spatiotemporal regulation from inside and outside the nucleus. *Genes & genetic systems*, *97*(1), 41–53. https://doi.org/10.1266/ggs.21-00065

43. Marchiani, S., Tamburrino, L., Muratori, M., Baldi, E. (2020). Spermatozoal Chromatin Structure: Role in Sperm Functions and Fertilization. In: Arafa, M., Elbardisi, H., Majzoub, A., Agarwal, A. (eds) Genetics of Male Infertility. Springer, Cham. https://doi.org/10.1007/978-3-030-37972-8_3

44. Simon, L. & Carrell, D.T. (2013) Sperm DNA Damage Measured by Comet Assay In Spermatogenesis: Methods and Protocols, Methods in Molecular Biology, Carrell, D. & Aston, K. (eds) Humana Press, Totowa, NJ. (Vol 927)

45. Ribas-Maynou, J., et al (2012). Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. Human Reproduction (Oxford, England), 27, 652–658. https://doi.org/10.1093/humrep/der461

46. Moretti, E., Cerretani, D., Noto, D., Signorini, C., Iacoponi, F., & Collodel, G. (2021). Relationship Between Semen IL-6, IL-33 and Malondialdehyde Generation in Human Seminal Plasma and Spermatozoa. *Reproductive sciences (Thousand Oaks, Calif.)*, *28*(8), 2136–2143. https://doi.org/10.1007/s43032-021-00493-7

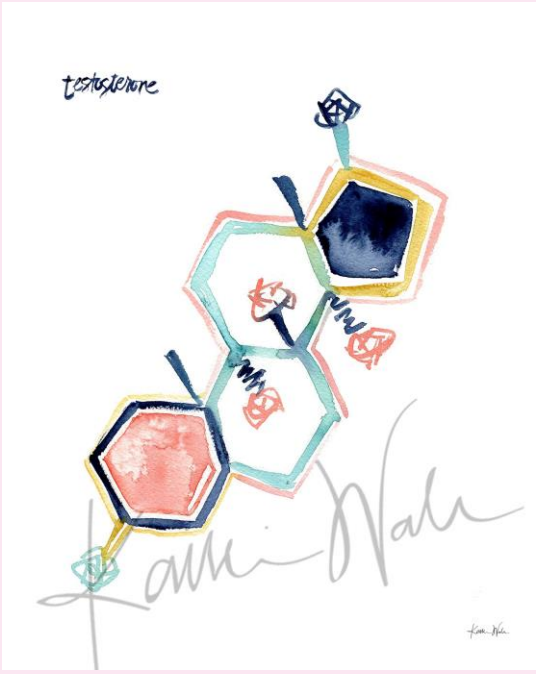
47. The jamovi project (2022). *jamovi*. (Version 2.3) [Computer Software]. Retrieved from https://www.jamovi.org.

48. Borges, E. Jr. *et al.* Sperm DNA fragmentation is correlated with poor embryo development, lower implantation rate, and higher miscarriage rate in reproductive cycles of non-male factor infertility. *Fertil. Steril.* **112**(3), 483–490 (2019).

49. Homa, S. T. *et al.* A comparison between two assays for measuring seminal oxidative stress and their relationship with sperm DNA fragmentation and semen parameters. *Genes* **10**(3), 236 (2019).

50. Anifandis, G. *et al.* COVID-19 and fertility: A virtual reality. *Reprod. Biomed. Online* **41**(2), 157–159 (2020).

51. *FSH abnormalities and male infertility*. Male Infertility Guide. (n.d.). https://www.maleinfertilityguide.com/fshabnormalities#:~:text=Elevated%20FSH%20Levels%20and%20male%20infertility&text=When%20the%20brain%20senses%20that,of%20the%20testicles%20is%20decreased (Access: August 3)



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ANNEXES

Annex 1. Objective 1 Summary of the results obtained by Mann-Whitney U Independent Samples T-Test. Mean ± SD; (P)-value: 0,05; NS: Not Significant

OBJECTIVE 1				
	N Mild	N Severe	T0 Mild	T0 Severe
Seminal volume (mL)	41	17	2.78 ± 1.19	2.16 ± 1.38
			NS	
Sperm concentration (Mz/mL)	41	17	66.56 ± 66.15	34.79 ± 50.24
			P = 0.022	
Total sperm (Mz)	41	17	193.89 ± 271.47	51.61 ± 81.35
			P = 0.002	
Motility type A (%)	41	17	16.58 ± 13.52	5.75 ± 7.44
			P = < .001	
Motility type B (%)	41	17	20.17 ± 13.43	11.37 ± 9.43
			P = 0.021	
Progressive motility (A+B) (%)	41	17	36.75 ± 21.68	17.11 ± 14.38
			P = 0.002	
Normal forms (%)	39	11	6.21 ± 6.63	7.45 ± 5.13
			NS	
Single-strand DNA fragmentation (%)	26	12	26.15 ± 11.39	46.76 ± 28.14
			P = 0.016	
Double-strand DNA fragmentation (%)	25	12	18.10 ± 9.22	36.30 ± 26.25
			P = 0.019	
IL6 seminal plasma levels (pg/mL)	9	9	38.53 ± 62.26	28.71 ± 18.91
			NS	
LH blood levels (U/L)	33	17	4.74 ± 2.25	3.76 ± 1.44
			NS	
FSH (U/L)	33	17	8.54 ± 7.27	8.74 ± 13.70
			NS	
INHB (ng/L)	29	17	158.52 ± 78.03	173.92 ± 60.90
			NS	
Testosterone (ng/dL)	33	17	432.84 ± 287.39	485.06 ± 208.66
			NS	

Annex 2. Objective 2 Summary of the results obtained by Wilcoxon W Paired Samples T-Test. The statistical analysis was conducted three times: all samples, only mild cases and only severe cases. Mean ± SD; (P)-value: 0 ,05; NS: Not Significant

OBJECTIVE 2								
Parameter	Sample group	N	T0	T3		N	T0	T6
Seminal volume (mL)	Total (Mild + Severe)	47	2.67 ± 1.33	2.45 ± 1.35	NS	23	2.30 ± 1.32	1.97 ± 1.04 NS
	Mild	33	2.89 ± 1.23	2.64 ± 1.27	NS	14	2.74 ± 1.16	1.94 ± 1.01 **P = 0.035**
	Severe	14	2.16 ± 1.46	2.01 ± 1.48	NS	9	1.61 ± 1.33	2.03 ± 1.14 NS
Sperm concentration (Mz/mL)	Total (Mild + Severe)	47	64.45 ± 66.22	63.18 ± 56.53	NS	23	51.09 ± 69.80	74.09 ± 72.52 NS
	Mild	33	80.02 ± 67.99	63.79 ± 62.10	NS	14	57.10 ± 77.42	50.66 ± 54.15 NS
	Severe	14	27.75 ± 45.50	61.76 ± 42.57	**P = 0.003**	9	41.74 ± 59.15	110.52 ± 85.11 **P = 0.020**
Total sperm (Mz)	Total (Mild + Severe)	48	174.91 ± 259.04	155.83 ± 180.53	NS	24	142.87 ± 318.31	145.03 ± 169.11 NS
	Mild	34	230.29 ± 287.12	165.98 ± 195.87	NS	15	202.57 ± 391.87	111.87 ± 139.99 NS
	Severe	14	40.43 ± 76.38	131.18 ± 139.70	**P = 0.030**	9	43.37 ± 68.90	200.29 ± 205.97 **P = 0.004**

Motility type A (%)	Total (Mild + Severe)	47	13.26 ± 13.65	P = 0.007	18.77 ± 14.18	23	13.47 ± 11.25	NS	15.64 ± 13.67
	Mild	33	17.28 ± 14.23	NS	20.98 ± 14.72	14	16.61 ± 11.78	NS	10.82 ± 9.83
	Severe	14	3.78 ± 4.79	P = 0.012	13.58 ± 11.67	9	8.59 ± 8.85	P = 0.004	23.14 ± 15.91
Motility type B (%)	Total (Mild + Severe)	47	19.43 ± 14.45	NS	22.72 ± 13.55	23	18.05 ± 12.48	NS	19.04 ± 11.82
	Mild	33	22.83 ± 14.76	NS	22.89 ± 14.01	14	20.50 ± 14.09	NS	15.17 ± 11.24
	Severe	14	11.41 ± 10.18	P = 0.011	22.33 ± 12.89	9	14.24 ± 8.88	P = 0.008	25.06 ± 10.58
Progressive motility (A+B) (%)	Total (Mild + Severe)	47	32.69 ± 23.65	P = 0.025	41.50 ± 20.90	23	31.52 ± 19.79	NS	34.68 ± 23.42
	Mild	33	40.11 ± 23.18	NS	43.87 ± 21.34	14	37.11 ± 21.12	NS	25.99 ± 18.58
	Severe	14	15.20 ± 13.70	P = 0.002	35.91 ± 19.40	9	22.84 ± 14.59	P = 0.004	48.20 ± 24.71
Normal forms (%)	Total (Mild + Severe)	36	7.19 ± 6.84	NS	6.82 ± 7.24	17	8.76 ± 7.02	NS	7 ± 5.67
	Mild	28	7.18 ± 7.36	NS	6.52 ± 7.98	12	7.83 ± 7.70	NS	7.25 ± 6.51
	Severe	8	7.25 ± 5.01	NS	7.88 ± 3.87	5	11 ± 5.05	NS	6.40 ± 3.36

Single-strand DNA fragmentation (%)	Total (Mild + Severe)	26	29.11 ± 20.28	NS	23.90 ± 13.26	15	30.32 ± 10.95	NS	30.17 ± 14.45
	Mild	19	22.55 ± 11.42	NS	21.76 ± 13.42	9	30.07 ± 10.92	NS	32.20 ± 13.50
	Severe	7	46.90 ± 28.60	NS	29.71 ± 11.78	6	30.69 ± 12.02	NS	27.13 ± 16.58
Double-strand DNA fragmentation (%)	Total (Mild + Severe)	28	22.95 ± 19	NS	17.89 ± 11.85	13	22.43 ± 12.30	NS	22.78 ± 9.43
	Mild	20	17.18 ± 9.25	NS	16.44 ± 12.06	8	18.66 ± 8.11	NS	23.74 ± 9.63
	Severe	8	37.37 ± 28.77	NS	21.50 ± 11.21	5	28.46 ± 16.27	NS	21.25 ± 10
IL6 seminal plasma levels (pg/mL)	Total (Mild + Severe)	16	25.11 ± 18.44	NS	31.78 ± 48.79	2	101.75 ± 136.97	NS	32.40 ± 30.83
	Mild	8	18.52 ± 17.66	NS	20.45 ± 18.16	1	198.60 ± NaN	NS	54.20 ± NaN
	Severe	8	31.69 ± 17.82	NS	43.11 ± 66.92	1	4.90 ± NaN	NS	10.60 ± NaN
LH blood levels (U/L)	Total (Mild + Severe)	30	4.14 ± 1.88	NS	3.81 ± 1.82	15	4.60 ± 2.05	NS	4.14 ± 1.62
	Mild	17	4.41 ± 2.18	NS	3.93 ± 2	7	5.52 ± 2.45	NS	4.80 ± 2.01
	Severe	13	3.78 ± 1.41	NS	3.66 ± 1.62	8	3.80 ± 1.30	NS	3.56 ± 0.98

FSH (U/L)	Total (Mild + Severe)	30	9.59 ± 11.78	11.06 ± 25.17	P = < .001	15	9.14 ± 14.52	5.13 ± 2.57	P = 0.026
	Mild	17	9.47 ± 8.37	15.91 ± 32.97	P = 0.028	7	6.31 ± 2.83	6.15 ± 2.69	NS
	Severe	13	9.74 ± 15.56	4.71 ± 2.12	P = 0.005	8	11.62 ± 19.99	4.25 ± 2.25	P = 0.008
INHB (ng/L)	Total (Mild + Severe)	28	165.36 ± 64.92	152.57 ± 59.51	NS	14	179.57 ± 72.03	159.73 ± 69.35	NS
	Mild	15	154.85 ± 72.80	147.23 ± 63.37	NS	6	208.75 ± 88.82	152.93 ± 91.94	NS
	Severe	13	177.48 ± 54.80	158.75 ± 56.61	NS	8	157.69 ± 52.18	164.82 ± 53.15	NS
Testosterone (ng/dL)	Total (Mild + Severe)	30	514.28 ± 265.85	487.57 ± 195.61	NS	15	491.65 ± 287.95	486.28 ± 252.22	NS
	Mild	17	525.49 ± 299.66	504.65 ± 224.83	NS	7	456.39 ± 323.46	443.17 ± 306.51	NS
	Severe	13	499.62 ± 225.09	465.23 ± 155.31	NS	8	522.50 ± 271.70	524 ± 207.88	NS