# Semen parameters, DNA fragmentation and sperm aneuploidy evaluation in the etiology of male infertility

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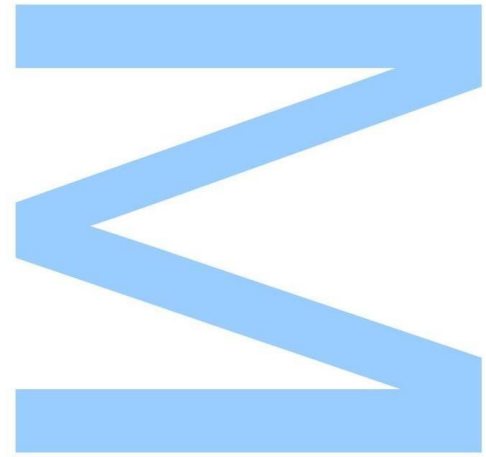

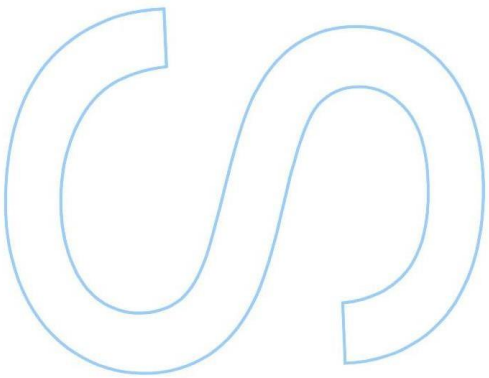

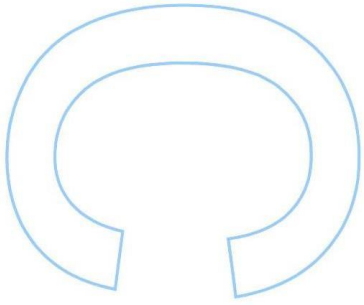

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# Resumo

Existem várias causas de infertilidade, tanto feminina como masculina. O estudo do casal infértil é de extrema importância para um diagnóstico correto e, consequentemente, um melhor tratamento. Na infertilidade masculina, a realização de estudos para além do espermograma podem revelar-se importantes, nomeadamente o estudo das aneuploidias e da fragmentação de DNA espermático. Apesar de alguns estudos já terem sido realizados para entender a sua utilidade clínica, a implementação destes testes na prática clínica ainda não foi realizada. Tendo isto em conta, o principal objetivo deste estudo foi tentar aclarar estas relações e avaliar se testes às aneuploidias e fragmentação de DNA espermático deveriam ser incluídos na investigação de rotina da infertilidade masculina. Além disso, procurámos estabelecer um valor *cut-off* a partir do qual uma diminuição significativa da qualidade espermática é observada, comprometendo a fertilidade masculina. Neste estudo, foram incluídos 835 indivíduos com indicação clínica de infertilidade (individual ou de casal) de 2007 a 2019. As amostras de sémen foram tratadas para avaliação dos parâmetros de espermograma, a fragmentação de DNA do esperma foi medida através da técnica *Terminal deoxynucleotidyl transferase dUTP nick-end labelling* (TUNEL) e as aneuploidias espermáticas usando a técnica Hibridação *in situ* de Fluorescência (FISH). A idade masculina parece estar relacionada com a fragmentação de DNA do esperma. O estudo da percentagem de fragmentação de DNA do esperma parece ser mais indicado em indivíduos oligozoospérmicos ou em indivíduos com anomalias associadas. Por outro lado, o estudo das aneuploidias espermáticas pode ser pertinente para indivíduos oligoteratozoospérmicos (OT) ou oligoastenoteratozoospérmicos (OAT). Foi ainda encontrada uma associação estatisticamente significativa e positiva entre a fragmentação de DNA e aneuploidias dos espermatozoides. Adicionalmente, um *cut-off* de 18.8% de fragmentação de DNA do esperma foi definido para a nossa população, usando a técnica de TUNEL por microscopia de fluorescência. Este estudo ajudou a compreender melhor a interação entre estes fatores masculinos e perceber em que casos devem ser realizados o estudo de aneuploidias ou fragmentação de DNA espermático para a investigação da causa de infertilidade masculina. Além disso, um novo valor *cut-off* foi definido para a fragmentação de DNA espermático a partir do qual o estado da fertilidade masculina pode ficar comprometido. Deste modo, o estudo poderá contribuir para um melhor aconselhamento reprodutivo para os casais inférteis.

# Abstract

There are several causes of infertility, either female or male. The study of the infertile couple is extremely important to achieve a correct diagnostic and, consequently, a better treatment. Considering male infertility, studies beyond sperm parameters seem to be important to be performed, such as sperm aneuploidies and sperm DNA fragmentation. Some studies were performed to understand the clinical utility of these DNA quality tests, however implementation in routine diagnosis have not yet been performed. Taking this into account, our main goal was to search for those relationships and evaluate if sperm aneuploidies and sperm DNA fragmentation should be included as valid tests in the routine investigation of male infertility. Additionally, we aimed to define a cut-off value above which significantly increased sperm DNA fragmentation can compromise male fertility. In this study, 835 individuals with clinical indication of infertility (individual or couple infertility) from 2007 to 2019 were included. Semen samples were investigated for conventional semen parameters, sperm DNA fragmentation assessed by Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) and sperm aneuploidies by using Fluorescence *in situ* Hybridization (FISH). Male age seemed to trigger sperm DNA fragmentation**.** Sperm DNA fragmentation analysis seems to be more indicated for oligozoospermic men or individuals with abnormalities in association. On the other hand, sperm aneuploidies testing could be pertinent for oligoteratozoospermic (OT) or oligoastenoteratozoospermic (OAT). A statistically significant and positive association between sperm DNA fragmentation and sperm aneuploidies was also found. Additionally, a cut-off point of 18.8% of sperm DNA fragmentation was established for our population, using TUNEL-assay by fluorescence microscopy. This study helped to better understand the interaction between these male factors and understand in which cases should be performed sperm aneuploidies or sperm DNA fragmentation tests for routine investigation of male infertility. Additionally, it allowed to recommend a new cut-off for sperm DNA fragmentation as the reference value above which male fertility status could be seriously compromised. This contributes for a better reproductive counselling to the infertile couples.

# Keywords

Male infertility, sperm DNA fragmentation, sperm aneuploidies, semen parameters, FISH, TUNEL, advanced paternal age, threshold value

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# Abbreviation List

A: Asthenozoospermic

ACMG: American College of Medical Genetics

APA: Advanced paternal age

ART: Assisted reproductive technology

AT: Asthenoteratozoospermic

ASRM: American Society for Reproductive Medicine

CHUSJ: São João University Hospital Center

DAPI: 4'6-diamidino-2-phenylindole

DNA: Deoxyribonucleic acid

dNTP: Dinucleotide Tri-Phosphate

DTT: 1,4-dithiothreitol

FET: Frozen Embryo Transfer

FISH: Fluorescence *in situ* Hybridization

HAI: Homologous Artificial Insemination

ICMART: International Committee for Monitoring Assisted Reproductive Technology

ICSI: Intracytoplasmic sperm injection

IM: Immotile

ISM: *in situ* motility

IUI: Intrauterine Insemination

IVF: *in vitro* Fertilization

NO: Normozoospermic

O: Oligozoospermic

OA: Oligoasthenozoospermic

OAT: Oligoasthenoteratozoospermic

OD: Oocyte Donation

OH: Hydroxyl

OI: Ovulation Induction

OT: Oligoteratozoospermic

PBS: Phosphate-buffered saline

ROC: Receiver Operating Characteristic

ROS: Reactive Oxygen Species

RPM: Rapid Progressive Motility

SCD: Sperm Chromatin Dispersion

SCSA: Sperm Chromatin Structure Assay

SD: Standard Deviation

SSC: Saline-sodium citrate

SPM: Slow Progressive Motility

SPSS: Statistical Package for Social Science

T: Teratozoospermic

TdT: Terminal deoxynucleotidyl transferase

TESA: Testicular Sperm Aspiration

TM: Total Motility

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick-end labelling

WHO: World Health Organization

# I. Introduction

## 1.1. Infertility and the influence of male factor

According to the World Health Organization (WHO) and the International Committee for Monitoring Assisted Reproductive Technology (ICMART) infertility can be defined as a failure to achieve a natural pregnancy after a year of regular and unprotected intercourse between an heterosexual couple [1]. Additionally, the Practice Committee of the American Society for Reproductive Medicine (ASRM) also enhanced the importance of sexual, medical and reproductive history of the couple in study. These and physical findings, can justify an earlier evaluation and treatment for women above 35 years old [2].

Infertility is not only a worldwide health issue but also a social issue. Social pressure to conceive is described by couples [3] and in fact, most of them, have reported to feel depression, anxiety, marital problems and loss of self-esteem [4, 5]. Globally, 15% of the couples have infertile problems [6]. From these, male factor can contribute from 20 to 70% of the cases, being 2 to 12% of men considered to be infertile. Inside of the global male factor contribution to the couple's infertility, European percentage is around 50% [7].

As male factors can be the reason why a couple cannot conceive a child, it is important to establish and understand which factors are involved, to achieve the best treatment approach. Genetical and pathological factors such as abnormal hormonal levels, varicocele, cystic fibrosis gene mutations, Y chromosome abnormalities, testicular cancer, epigenetic errors, pituitary tumours or even idiopathic factors have been associated with male infertility [2, 8]. However, it is believed that male infertility can also be influenced by environmental, occupational and lifestyle factors, compromising semen quality [9]. In fact, studies have already reported that factors such as cigarette smoking, alcohol, obesity, radiation, genital heat stress, dietary practices or the use of illicit drugs can contribute to an increasing deterioration in human sperm quality and sperm aneuploidy [9, 10]. These are all factors that can be altered with healthier lifestyle choices, helping overcome its negative effect in semen quality [9].

## 1.2. Semen analysis and advanced male age

In clinical practice, semen analysis is required and almost an obligatory test in the research of a couple's infertility problem, as it can give relevant information about the quality and semen production [11]. Sperm concentration/mL, sperm motility and the percentage of spermatozoa with normal morphology are some of the parameters evaluated and the most important ones when describing the male fertility status [12].

According to the WHO guidelines [13], normal semen parameters are characterized by a sperm concentration above 15 million/mL, at least 32% of sperm with progressive motility and the presence of more than 4% of sperm with a normal morphology. These are the values expected to be necessary to achieve a normal pregnancy naturally [2] .

In the presence of abnormal values, men are divided into different classification groups [14]. Indeed, men can be classified as oligozoospermic (O), when its semen sample has a reduced concentration of sperm cells (below 15 million/mL), asthenozoospermic (A), when the percentage of sperm progressive motility is below 32% and teratozoospermic (T), when the percentage of sperm morphologically normal is below 4%. When more than one of the semen parameters have abnormal values, a more complex fertility status is present [13]. However, semen analysis is a poor predictor of male fecundity, once infertile men can have normal semen parameters values and, consequently, be classified as normozoospermic (NO) [11, 15]. In fact, a study with the purpose of understanding the relationships between semen analysis and the capacity to conceive a child have reported that, in a population of subfertile couples, 41% of the men had normal values of all semen parameters, according to WHO criteria [16]. Albeit in this report, the authors often use the word "subfertility", it is important to highlight that the definition of subfertility is presently considered, according to ICMART and the ASRM, a "term that should be used interchangeably with infertility", once a different degree of fertility status is not express between these two terms [17]. Nevertheless, it is noticeable that following only WHO criteria may not be enough to predict and distinguish the individuals that are more likely to conceive a child from the ones who are not. Although semen analysis is important to understand men fecundity status, it is not sufficient for its diagnosis.

Advanced paternal age (APA) is one of the main studied factors in infertility that cannot be defeated. In the last few decades, paternal age has been increasing. This delayed fatherhood, mainly due to socioeconomic factors such as career, educational

goals or financial stability [18], is believed to compromise the achievement of a natural pregnancy [19]. Although a consensus definition of APA between clinics has not been achieved, the American College of Medical Genetics (ACMG) has establish its threshold at 40, at the time of conception [20]. In fact, most sperm banks only accept donors with ages up to 39 years [21], once APA has been already described as an increasing factor for the risk of developmental of psychiatric disorders such as epilepsy, autism spectrum disorder, intellectual disability or schizophrenia [22-25].

Several studies have been done to understand the influence of the paternal age in semen parameters, once semen analysis is normally the first test performed. Some studies did not find any significant correlation between sperm parameters and APA [26, 27]. Others have found significant associations but is not consensual which semen parameters can be compromised by male age. For example, some authors reported that with increased age, there is an increased sperm concentration, a significantly decline in semen volume and vitality but no other significant associations with the others parameters [28]. Others say that, with increased male age, it is only observed significant differences in semen volume, semen viscosity and motility [29]. Many other studies tried to establish a relationship between male age and semen parameters, but incongruences between studies remain [30-36]. These variances between studies can be due to different sample sizes, its heterogeneity and amplitude of age range.

Taking all this into account, it becomes relevant to study sperm DNA quality, to improve infertile men diagnosis. In fact, sperm DNA fragmentation and/or sperm aneuploidies tests might give complement information about fertility status [37-39]**.** Many studies have been performed to comprehend the clinical utility of these DNA quality tests, however implementation in routine diagnosis have not yet been performed.

## 1.3. Sperm DNA fragmentation

Sperm DNA fragmentation has been studied as a possible defect in spermatozoa that can compromise male fecundity [40] and can be defined as a damaged or denatured DNA that is not able to be repaired [41].

This damage can occur due to a wide range of factors. *Pacey et al.*, have reviewed the lifestyle and environmental factors that can be related with increased sperm DNA fragmentation and they have proposed that these factors can be subdivided into chemical agents, namely pesticides, smoke and pollution are included; physical agents,

including heat and radiation; and biological factors, such as diabetes, male age, body mass index, and sexually transmitted infections [42]. All these factors mentioned, along with abortive apoptosis, overproduction of reactive oxygen species (ROS), varicocele and defects during the process of spermatogenesis can make sperm more vulnerable to DNA fragmentation [42, 43]. However, most of the times, the cause that trigger this injury is unknown [44].

As it was mentioned before, male age is a biological non-modifiable factor that is believed to be related with DNA damage. In fact, most of the studies found that fragmentation in spermatozoa DNA can significantly increase with male age [26, 29, 45-47]. The main theory of this association relies on the overproduction of ROS and errors during spermatogenesis that older men are more prone to [48] and increased levels of ROS have already been reported to be present with increased sperm DNA fragmentation [49, 50]. Notwithstanding, a minor group of studies did not find any association between sperm DNA fragmentation and male age [28, 51].

To include sperm DNA fragmentation testing in the routine diagnosis of male infertility, it is important to evaluate if it is an informative parameter and if adds value to the male fertility status. For that, studies comparing both tests, sperm DNA fragmentation and semen parameters, are needed. *Ganzer et al.* investigated this correlation in a population of infertile patients (N = 1562). Results of this study have accomplished that semen parameters were negatively associated with sperm DNA fragmentation [52]. Other similar studies have found identical results [53-55]. Additionally, associations were found with asthenozoospermia [56] and with teratozoospermia [57]. In fact, *Brahem et al.* studied if there was significant differences of sperm DNA fragmentation in men with isolated teratozoospermia, comparing to a fertile population, and found a positive and significant correlation between sperm DNA fragmentation and different types of morphologically abnormal spermatozoa [57]. Other groups have also focused in this issue and their results corroborate these findings [58, 59].

However, a study performed in an infertile men population (N = 318) to evaluate the association between sperm DNA fragmentation and routine semen analysis did not find a strong correlation between both, and suggested that sperm DNA fragmentation tests should be only performed as an additional test in men with risk factors, such as smoking, APA or alcohol consumption [60].

Additionally, viability is one of the parameters included in semen analysis and its association with sperm DNA fragmentation has been investigated. Viability refers to the percentage of living sperm in the semen sample and is estimated by measuring the membrane integrity of sperm cells [13]. With the membrane integrity compromised, DNA

of the sperm can be more prone to damage and, consequently, to fragmentation [61]. Based on this theory, investigators have searched for a relationship between them, speculating if poor viability can be a good predictor of high levels of sperm DNA fragmentation. In fact, significant and strong associations between viability and sperm DNA fragmentation have been found, and suggested that, depending on the percentage of sperm viability, sperm DNA fragmentation testing may not be required [61]. However, others did not find this correlation and more studies have to be done to corroborate either of them [60].

Sperm DNA fragmentation could be measure by different methods subdivided into direct and indirect. Direct measurements directly measure the DNA fragmentation with the use of probes or dyes. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) or Comet assay are examples of direct techniques. The methods that only measure the susceptibility of the DNA to be denatured are called indirect methods being Sperm Chromatin Structure Assay (SCSA) and Sperm Chromatin Dispersion test (SCD) two examples [62, 63]. As different techniques can be used to measure sperm DNA fragmentation, a unique threshold value is not possible to be defined [64] and standardization between laboratories is almost impossible to be achieved.

Due to its better accuracy, methods with direct measurements are preferable [63] and as it was mentioned before, TUNEL is one of them. TUNEL is a direct measurement DNA fragmentation technique where DNA chain nicks can be identified by labelling the free terminal 3'-hydroxyl (OH) with modified nucleotides in an enzymatic reaction. With this reaction, fragmented spermatozoa DNA could be recognized using a fluorescent or non-fluorescent dye. These measurements can be done by optic or fluorescent microscopy, or by flow cytometry [65]. TUNEL technique advantages are the ability to detect DNA breaks on single or double strands, the capacity to be performed on frozen or fresh samples and even on only few sperm [43]. However, different threshold values should be considered, whether the measurement of fragmented DNA is performed by flow cytometry or by fluorescence microscopy. Although the principle is the same, there are differences between both. In fact, a study performed to understand those reported that, in average, flow cytometry can detect positive results 2.6 times higher than fluorescence microscopy [66]. Knowing this, a single threshold value for both flow cytometry and fluorescence microscopy should not be used.

There are studies in the literature that evaluated sperm DNA fragmentation using flow cytometry TUNEL. Nevertheless, different threshold values were described [67-70], which seems to be related to different sample sizes or due to high heterogeneity between populations. Regarding sperm DNA fragmentation using fluorescence microscopy

TUNEL, only few studies were published in the literature [71-73]. Moreover, some authors defend that, as a standardization of the protocols is still missing, for a more accurate diagnosis, a cut-off value should be defined for each laboratory, taking into account the sperm DNA fragmentation method used and patients [74].

Sperm DNA fragmentation can compromise male fecundity and influence both natural and medically assisted reproduction. However, its incorporation in the routine diagnostic investigation of male infertility has not yet been performed once no standardization exists between laboratories. It is important also to define a cut-off value above which male fertility is compromised.

## 1.4. Sperm aneuploidy

Sperm aneuploidy is also recognized as one of the genetic factors that can lead to infertility and its influence on the male reproductive outcome has been studied for more than forty years [75]. Briefly, spermatogenesis is a process in which mature spermatozoa are formed. Spermatogonia (diploid cells) undergo mitosis since fetal life. Later, when puberty begins, these diploid cells undergo meiosis. After two meiotic divisions, an haploid cell (spermatid) is formed with posterior differentiation, thus forming a mature spermatozoon [76]. However, during these cell divisions, errors can happen and an incorrect division of the chromosomes can occur, giving rise to a cell with an abnormal structural or number of chromosomes [77]. In the present study, we focused on sperm aneuploidies.

By definition, chromosome aneuploidy is the existence of an abnormal number of chromosomes in a cell and most of the times is related with risk of miscarriage, cognitive impairment and fetal anomalies [41, 78, 79]. Aneuploidies are very common in spontaneous abortions, namely in the first trimester of pregnancy. Aneuploidies of the 13, 18, 21, X and Y chromosomes are compatible with a term pregnancy so, in the investigation of a male infertility, the study have been focused into these chromosomes. [39, 80].

As it was mentioned before, it has been questioned the influence of APA in semen parameters and sperm DNA fragmentation. Regarding sperm aneuploidy, the question remains the same. Its influence in sperm chromosomal abnormalities is still not consensual. In spite of many of these studies did not show this effect of male age in sperm aneuploidy [81, 82], there are reports that have shown its significant influence [26,

83, 84]. In fact, along with other authors, *Brahem et al.* found in infertile patients that sperm diploidy increased with age, findings not observed in fertile patients [28].

Besides male age factor, environmental, clinical and biological factors may influence the rate of sperm aneuploidies [10] and it has been questioned if sperm aneuploidies are associated with specific groups of infertile men. For that, associations between abnormal semen parameters and sperm aneuploidy rates were investigated. In fact, some studies proposed an association between different severities of oligozoospermia and aneuploidy rates [85-87]. However, associations between sperm motility, sperm morphology and aneuploidy rates were not always consensual [86, 88, 89], although some research groups have claimed to find a relation between these variables. For example, *Petousis et al.* have shown a significant correlation between asthenozoospermia and aneuploidies, especially for chromosomes 13 and 15 [90]. Similar results are visible in other investigations [91, 92]. In this matter, it is important to investigate and identify whether specific groups of infertile men are more prone to produce sperm aneuploidies in order to facilitate the identification of the fertility problem and the best method for its treatment.

To detect sperm aneuploidies, Fluorescence *in situ* hybridization (FISH) is the most used method. Briefly, FISH is a molecular cytogenetic technique that uses specific DNA probes, with different fluorochromes each, for the identification of specific sequences of DNA in human chromosomes. With decondensed spermatozoa heads, it is possible to analyse the DNA content of thousands of spermatozoa in just one semen sample using fluorescence microscopy [93, 94]. Although this method is fast and easy to perform, this technique has some problems. Some reports have suggested frequency ranges of disomy for chromosomes 13, 18, 21, X and Y in normal fertile men [95, 96]. Once these are not always homogenous, comparisons may be difficult to be performed [97]. In fact, a standardization of FISH results analysis is missing, being one of the reasons why sperm aneuploidy screening is not yet incorporated in the clinical diagnosis for male infertility [78, 80].

## 1.5. Sperm DNA fragmentation vs Sperm aneuploidies

As two independent genetic tests for semen quality, sperm DNA fragmentation and sperm aneuploidies have been compared in the last few years.

In fact, studies were performed with the aim of understanding if exists any correlation between them. If the presence of one could condition or trigger the other. Many studies have found significant and positive associations between sperm DNA fragmentation and sperm aneuploidy in infertile men. Although some have found this association only for total aneuploidy levels [98, 99], others have observed an increased fragmentation level in sperm cells with sex chromosome aneuploidies [100]. There were also authors who claimed that, taking into account only sex chromosomes, the sperm DNA fragmentation level was significantly higher when Y chromosome is present in spermatozoa [101]. Nevertheless, mechanisms that could explain this association are not always clear and many hypotheses have been proposed by different investigators [99, 100, 102]. In contrast to these studies, there are researchers that did not find any significant correlations between these two variables [103, 104].

In order to compare studies and obtain informative results, the population studied must be similar and the use of same laboratory techniques is crucial. To our knowledge, few studies using FISH and TUNEL techniques, compared sperm aneuploidy frequencies and sperm DNA fragmentation in infertile men [98, 99].

# II. Objectives

The main goal of this study was to evaluate if sperm aneuploidies and sperm DNA fragmentation should be included as valid tests in the routine diagnostic investigation of male infertility. For that, comparisons between semen parameters, male age, sperm aneuploidies and sperm DNA fragmentation were performed. Additionally, we aimed to define a cut-off value above which significantly increased sperm DNA fragmentation observed can compromise male fertility.

With this study we expected to make clear relevant information in order to contribute for a better reproductive counselling to the infertile couple.

# III. Methods

## 3.1. Patients, Sample Collection and Semen analysis

Retrospective and prospective investigation was made, with a twelve-year period considered, from April 2007 to December 2019. A total of 835 individuals with clinical indication of infertility were included in this study. Sample collection and semen analysis were performed at Centre for Reproductive Genetics Prof. Alberto Barros and at São João University Hospital Center (CHUSJ), Porto, Portugal. Semen collection was performed by masturbation after 3-5 days of sexual abstinence, into sterile containers. After liquefaction, semen analysis was performed following WHO guidelines.

## 3.2. Genetic tests

This study was mostly retrospective. Nevertheless, all the studies performed since the beginning of this research were also included in this investigation, so this work is also a prospective study.

### 3.2.1. Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL)

For sperm DNA fragmentation evaluation, TUNEL-assay was performed, with *In Situ* Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany). Semen sample collection was performed by masturbation after a period of 3 days of abstinence. Before starting the TUNEL technique, samples had to be prepared. Samples were centrifuged (20 min, 1500rpm), seminal liquid removed and then washed with phosphate-buffered saline buffer (PBS, Sigma, Barcelone, Spain) at 37ºC, for 10 min at 1500rpm. Supernatant was removed and sperm concentration adjusted by adding PBS. A smear was done and the slides were left to air dry. For the TUNEL-assay procedure cells were then fixed with 4% paraformaldehyde/PBS (Sigma, Barcelone, Spain) for 1h at room temperature. After that, permeabilization of the membrane with 0,1%Sodium Citrate/0,1%Triton-X (Sigma, Barcelone, Spain) was performed (2 min, 4ºC) in order to enable DNA observation. After washes with PBS (2x 5min), the slides were incubated with 50 µl of labelling solution (5µl of the terminal deoxynucleotidyl transferase (TdT) enzyme and 45µl of dNTPs labelled with a green fluorochrome). This occurred at 37ºC in a dark moist chamber for 60 minutes. After incubation, slides were washed 4 times

and counterstained with Vectashield antifade medium containing 4'6-diamidino-2-phenylindole (DAPI; Vector Laboratories, U.S.A.). DAPI is a specific fluorescent dye that repels water and is used to find sperm cells once it binds to DNA. About 1000 sperm were evaluated, per sample, in a fluorescent microscope (Axio Imager Z1, Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) fitted with a CCD camera (AxioCam MRm, Zeiss) and an automated image software (FISH Imaging System, version 5.1, MetaSystems GmbH, Altlussheim, Germany). Positive TUNEL results showed a green fluorescence whereas negative TUNEL results had no fluorescence (Fig. 1). According to this, the percentage of spermatozoa with fragmented DNA was calculated. For each patient, the procedure was done twice and in different days. Mean percentage of the two results was then calculated.

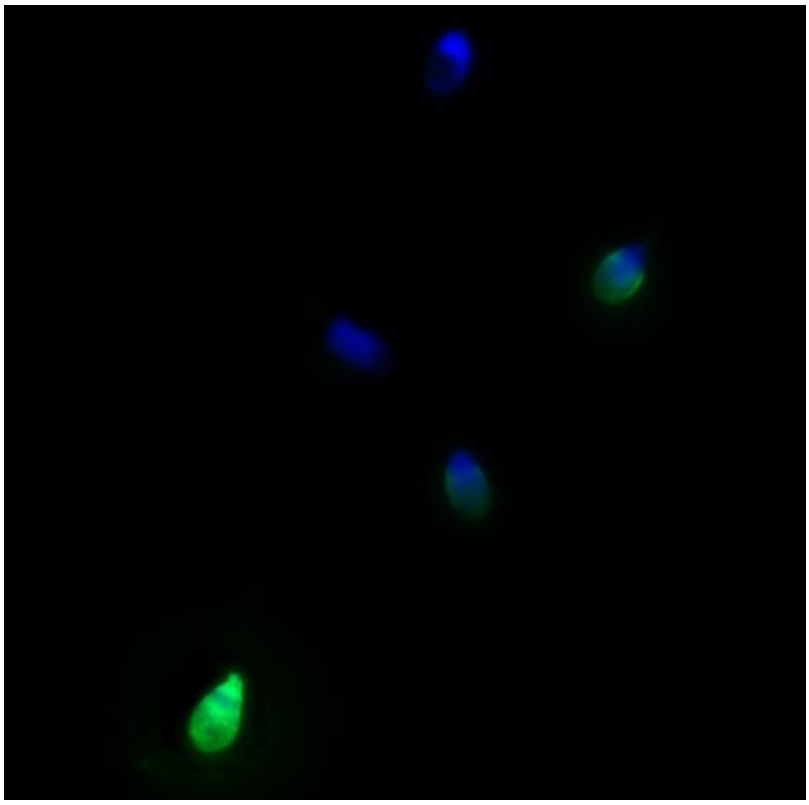

**Figure 1.** Image from a TUNEL result. (Green fluorescence – DNA fragmented, Blue fluorescence – normal DNA).

### 3.2.2. Fluorescence *in situ* Hybridization (FISH)

For sperm aneuploidies, FISH technique was used, with the *AneuVysion Multicolor DNA Probe Kit* (Abbott, Illinois, U.S.A.). Semen sample collection was made by masturbation after a period of 3 days of abstinence. After liquefaction (30 minutes, 37 ºC), samples were centrifuged (20 minutes, 1500rpm) and seminal liquid removed. PBS was added at 37 ºC and the sample was left at the same temperature for 10 minutes. After two washes (10 min, 1500rpm), semen samples were fixed with methanol:acetic acid (3:1; VWR International, Stockholm, Sweden/Panreac, Barcelona, Spain), washed twice (2x 10, 1500rpm) and kept at -20ºC until the FISH procedure. FISH was performed for chromosomes 13, 18, 21, X and Y using *AneuVysion Multicolor DNA Probe Kit*. For each patient, two slides were used. One for chromosomes 13 and 21 analysis (labelled with green and red, respectively) and the other for chromosomes 18, X and Y analysis (labelled with blue, green and red, respectively). Thus, samples were spreaded in slides and washed 2 times for 3 minutes each with saline-sodium citrate buffer (SSC; Invitrogen, Scotland, UK; 1:10) and then dehydrated with ethanol (70%, 96% and 100%) for 2 minutes each. In order to reach the DNA, sperm heads were decondensed with 1,4-dithiothreitol (DTT, Roche Applied Systems, Penzberg, Germany) solution at 37ºC and after washes with 2xSCC and dehydrations. DNA denaturation occurred by immersing slides in a 70% formamide solution at 73ºC for 5 minutes. Probe mixture was added (5 µL) in a coverslip and applied in the selected cell region. Parafilm was used to seal the coverslip and hybridization occurred at 37ºC overnight, in a humidified chamber. DAPI was again used to counterstain DNA and about 1000 spermatozoa were observed and analyzed in a fluorescent microscope. For each sperm cells, the number of fluorescent signals observed was counted, taking into account the color fluorescence emitted from each chromosome. For example, two distinguishable red signals in one single sperm cell, it is counted as a disomy of chromosome Y (Fig. 2). According to this, the percentage of aneuploidy and disomy of each chromosome was calculated.

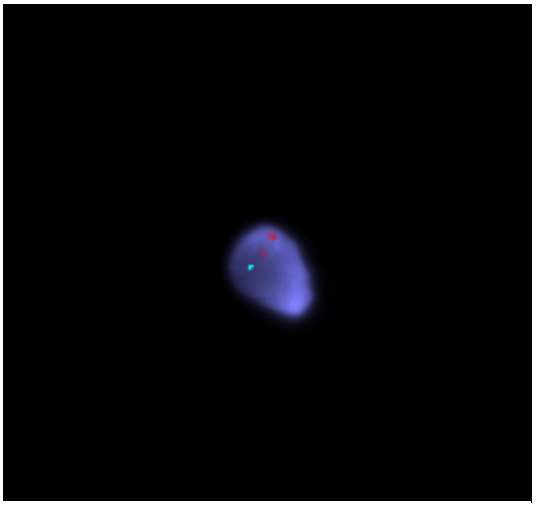

**Figure 2.** Image from a FISH result for chromosomes Y (red, 2 signals) and 18 (blue, 1 signal).

## 3.3. Elaboration of a database

A database with all the relevant information for this study was elaborated. This database included date of sample collection, percentage of sperm DNA fragmentation, euploidies, aneuploidies and individual disomies of chromosomes 13, 18, 21, X and Y percentages. Percentage of Total Disomy was also included and calculated by adding all the individual disomies and dividing them for the total of spermatozoa counted in the sample. Date of birth and respective male age when the tests were done were also included. All the semen parameters available, when semen analysis was performed, such as volume, concentration/mL and total concentration, multiplying volume by concentration/mL were included. Rapid progressive motility (RPM), slow progressive motility (SPM), in situ motility (ISM) and immotile (IM) are part of motility classification of the spermatozoa and they were taking in count. Total Progressive Motility was after calculated adding RPM and SPM, and total motility (TM) adding ISM to Total Progressive Motility. Vitality, hypoosmolality and pH were also included in this database as well as the classification of each individual semen analysis, according to WHO 2010 guidelines. Oligozoospermia was considered when concentration/mL was below 15 million, asthenozoospermia when total progressive motility was under 32% and teratozoospermia was classified when morphology had percentages lower than 4. Normozoospermia was considered when values were above each reference of these semen parameters. Only men with normal karyotype (46, XY) were included in this study.

Since it was difficult to have a control group (fertile men with fertility proved, with at least a child), all the NO patients with a child born or when the fertility treatments were done due to a female factor was selected from our database. This group included 60 individuals.

This study was approved by the Ethical Committee of the CHUSJ (Nº 412/19). The database anonymization was done by giving to each patient a random number following the guidelines of the Ethical Committee of CHUSJ.

## 3.4. Statistical Analysis

The statistical analysis of the data was performed using the program IBM Statistical Package for Social Science (SPSS) Statistics version 26 (IBM Corp, Armonk [NY], US). Normality of variables was assessed by inspecting the symmetry coefficient and observation of the distribution. As the sample size was moderately high, the central limit theorem ensures that the mean follows a normal distribution. When the distribution was asymmetrical with a right tail, a logarithm transformation of the variable was performed. Depending on data, two main tests were used. Pearson's product-moment correlation and Spearman's rank correlation. Each has a correlation coefficient ($\rho$) that ranges from -1 to 1 in order to evaluate the type of relationships between variables.

For comparisons between infertile men groups of sperm DNA fragmentation and sperm aneuploidies, after Bonferroni correction, Dunn test was used to observe if there was any relevant group that is distinguished from the other.

To determine the sperm DNA fragmentation cut-off value, the area under the receiver operating characteristic (ROC) curve was calculated, along with its sensitivity and specificity. Youden Index was used to find a threshold that maximizes both sensitivity and specificity.

Statistical significance was established at $p < 0.05$, while a P-value superior to 0.05 was considered statistically not significant.

SPSS version 26 or Microsoft Office Excel 2019 was used to represent graphics and tables.

# IV. Results

## 4.1. Population Study

A total of 835 male patients with clinical indication of infertility (individual or couple infertility) were studied between 2007 and 2019. Semen analysis was performed for all patients. Taking into account these results and following the WHO 2010 guidelines, patients were then divided in two categories: NO (N = 286) and non-normozoospermic (N = 549). The non-normozoospermic group were divided in subcategories: 44 were asthenozoospermic (A), 38 oligozoospermic (O), 166 teratozoospermic (T), 24 oligoasthenozoospermic (OA), 54 oligoteratozoospermic (OT), 70 asthenoteratozoospermic (AT), and 153 oligoasthenoteratozoospermic (OAT). From all these patients, 809 were recommended for the study of sperm DNA fragmentation and 303 for the study of sperm aneuploidies. About 277 of the patients performed both tests. From the NO group, 60 individuals were selected as the control group (see material and methods). Moreover, 588 went through Intracytoplasmic Sperm Injection (ICSI) treatments, 102 through *in vitro* Fertilization (IVF), 14 Ovulation Induction (OI), 7 Frozen Embryo Transfer (FET), 5 Homologous Artificial Insemination (HAI), 3 Testicular Sperm Aspiration (TESA), 2 Oocyte Donation (OD), 1 Intrauterine Insemination (IUI) and 113 information was not available.

A descriptive analysis was performed for each variable studied and for the whole population (Table 1).

**Table 1.** Variables studied, total number of patients in each sample, its mean, standard deviation (SD) and range.

| Variable | Total (N) | Mean ± SD | Range (Min-Max) |
|---|---|---|---|
| **Sperm DNA fragmentation (%)** | 809 | 21.38 ± 11.682 | 1.1 – 87.2 |
| **Total Sperm Aneuploidy (%)** | 303 | 0.87 ± 1.196 | 0 – 15.9 |
| **Disomy 13 (%)** | 303 | 0.15 ± 0.210 | 0 – 2.25 |
| **Disomy 21 (%)** | 303 | 0.17 ± 0.194 | 0 – 1.6 |
| **Disomy 18 (%)** | 303 | 0.16 ± 0.262 | 0 – 2.69 |
| **Disomy XX (%)** | 303 | 0.05 ± 0.144 | 0 – 2 |
| **Disomy XY (%)** | 303 | 0.25 ± 0.322 | 0 – 2.8 |
| **Disomy YY (%)** | 303 | 0.11 ± 0.163 | 0 – 1.20 |
| **Total Sperm Disomy (%)** | 303 | 0.45 ± 0.436 | 0 – 3.77 |
| **Male age** | 835 | 36.63 ± 5.774 | 17 – 62 |
| **Sperm Concentration (x10$^6$/mL)** | 834 | 53.35 ± 62.089 | 0.004 – 487.5 |
| **Total Progressive Motility (%)** | 835 | 40.17 ± 20.626 | 0 – 96 |
| **Morphology (%)** | 835 | 4.21 ± 3.848 | 0 – 23 |
| **Vitality (%)** | 761 | 71.44 ± 14.871 | 2 – 98 |
| **Hypoosmolality (%)** | 692 | 62.79 ± 14.864 | 8 – 93 |

## 4.2. The male age relevance in the infertile male population

As age can't be changeable, it is important to understand if a non-modifiable factor has significant influence in semen parameters, sperm DNA fragmentation and sperm aneuploidies. A relationship between increased male age and abnormal semen parameters was investigated. There was a statistically significant correlation between male age and concentration/mL ($\rho$ = 0.082, P = 0.01), vitality ($\rho$ = -0.177, P < 0.001) and hypoosmolality ($\rho$ = -0.140, P < 0.001). No significant differences with total progressive motility and morphology were found. Comparing male age of the patients with sperm DNA fragmentation, a statistically significant positive association was found ($\rho$ = 0.192, P < 0.001). However, significant correlations with sperm aneuploidies and total sperm disomy with male age were not found (Table 2). Only associations with individual disomies for chromosomes 13 ($\rho$ = 0.131, P = 0.02) and X ($\rho$ = 0.121, P = 0.03) were statistically significant (Table 3).

**Table 2.** Associations between male age with semen parameters, sperm DNA fragmentation and total sperm aneuploidy and its corresponding P-value and *ρ*. Statistically significant results are highlighted in bold.

| | | Male Age |
|---|---|---|
| **Concentration** | P-value | **0.01** |
| | *ρ* | 0.082 |
| **Total Progressive Motility** | P-value | 0.74 |
| | *ρ* | -0.011 |
| **Morphology** | P-value | 0.14 |
| | *ρ* | 0.051 |
| **Sperm DNA fragmentation** | P-value | **< 0.001** |
| | *ρ* | 0.192 |
| **Vitality** | P-value | **< 0.001** |
| | *ρ* | -0.177 |
| **Hypoosmolality** | P-value | **< 0.001** |
| | *ρ* | -0.140 |
| **Total Sperm Aneuploidy** | P-value | 0.9259 |
| | *ρ* | -0.005 |

**Table 3.** Associations between male age with total sperm disomy, disomy 13, 21, 18, XX, XY and YY and its corresponding P-value and *ρ*. Statistically significant results are highlighted in bold.

| | | Male Age |
|---|---|---|
| **Total Sperm Disomy** | P-value | 0.829 |
| | *ρ* | 0.012 |
| **Disomy 13** | P-value | **0.02** |
| | *ρ* | 0.131 |
| **Disomy 21** | P-value | 0.613 |
| | *ρ* | 0.029 |
| **Disomy 18** | P-value | 0.81 |
| | *ρ* | 0.014 |
| **Disomy XX** | P-value | **0.03** |
| | *ρ* | 0.121 |
| **Disomy XY** | P-value | 0.55 |
| | *ρ* | 0.030 |
| **Disomy YY** | P-value | 0.63 |
| | *ρ* | 0.027 |

## 4.3. Semen analysis and sperm DNA testing

In order to give a more accurate information about men fertility status, semen analysis, sperm DNA fragmentation and sperm aneuploidies tests were studied.

Statistically significant and negative correlations were found between sperm DNA fragmentation and sperm concentration ($\rho$ = -0.221, P < 0.001), total progressive motility ($\rho$ = -0.394, P < 0.001) and morphology ($\rho$ = -0.238, P < 0.001) (Table 4). Correlations with vitality and hypoosmolality were also found ($\rho$ = -0.553, P < 0.001; $\rho$ = -0.493, P < 0.001, respectively) (Table 5).

Associations between sperm aneuploidies and semen parameters were also investigated. Table 4 shows the correlations between total sperm aneuploidy and total sperm disomy with semen parameters. Statistically significant negative associations were found between total sperm aneuploidy with concentration ($\rho$ = -0.366, P < 0.001), total progressive motility ($\rho$ = -0.216, P < 0.001) and morphology ($\rho$ = -0.158, P = 0.006). Total sperm disomy was also correlated with concentration, total progressive motility and morphology ($\rho$ = -0.278, P < 0.001; $\rho$ = -0.175, P = 0.002; $\rho$ = -0.142, P = 0.01, respectively).

**Table 4.** Correlations, P-values and *ρ* between sperm DNA fragmentation, total aneuploidies, total disomy and with semen parameters. Statistically significant results are highlighted in bold.

| | | Concentration | Total Progressive Motility | Morphology |
|---|---|---|---|---|
| **Sperm DNA fragmentation** | P-value | **< 0.001** | **< 0.001** | **< 0.001** |
| | *ρ* | -0.221 | -0.394 | -0.238 |
| **Total Sperm Aneuploidy** | P-value | **< 0.001** | **< 0.001** | **0.006** |
| | *ρ* | -0.366 | -0.216 | -0.158 |
| **Total Sperm Disomy** | P-value | **< 0.001** | **0.002** | **0.01** |
| | *ρ* | -0.278 | -0.175 | -0.142 |

**Table 5.** Correlations, P-values and *ρ* between sperm DNA fragmentation with vitality and hypoosmolality. Statistically significant results are highlighted in bold.

| | | Vitality | Hypoosmolality |
|---|---|---|---|
| **Sperm DNA fragmentation** | P-value | **< 0.001** | **< 0.001** |
| | *ρ* | -0.553 | -0.493 |

## 4.4. Infertile groups and its relation with sperm damage

After analysed possible correlations between sperm DNA fragmentation/sperm aneuploidies with semen parameters, it would be relevant to investigate what groups of individuals could really benefit from sperm DNA fragmentation or sperm aneuploidy testing.

So, differences between the normozoospermic and non-normozoospermic groups for the presence of sperm DNA fragmentation, total sperm aneuploidy and total sperm disomy were observed. Table 6 shows the mean and standard deviation (SD) of each group and corresponding P-value. Significant statistically differences between these groups were found for sperm DNA fragmentation ($P < 0.001$), total sperm aneuploidy ($P = 0.007$) and total sperm disomy ($P = 0.016$).

**Table 6.** Comparisons between non-normozoospermic and normozoospermic groups for the presence of Sperm DNA fragmentation (%), Total Sperm Aneuploidy (%) and Total Sperm Disomy (%) and corresponding mean, SD and P-value. Statistically significant results are highlighted in bold.

| | **Non-normozoospermic** | | **Normozoospermic** | | |
|---|---|---|---|---|---|
| | **Mean** | **SD** | **Mean** | **SD** | **P-value** |
| **Sperm DNA fragmentation (%)** | 23.72 | 12.546 | 16.90 | 8.231 | **< 0.001** |
| **Total Sperm Aneuploidy (%)** | 1.02 | 1.501 | 0.65 | 0.365 | **0.007** |
| **Total Sperm Disomy (%)** | 0.50 | 0.526 | 0.38 | 0.225 | **0.016** |

After comparisons between both groups (non-normozoospermic and NO), differences could also be present in the sub-categories established.

For sperm DNA fragmentation, statistically significant differences between the NO group and the O (P = 0.002), OT (P < 0.001), AT (P < 0.001), OAT (P < 0.001) groups are shown in table 7. The percentage of sperm DNA fragmentation is significantly lower in the NO group (16.90 ± 8.231; Figure 1). Also, statistically significant lower values were found in the group T (19.52 ± 9.363) when compared with AT and with OAT (27.04 ± 11.760, P < 0.001 and 26.54 ± 14.373, P = 0.001, respectively; Figure 3).

**Table 7.** Comparisons of sperm DNA fragmentation between groups taking into account semen parameters.

| | **NO** | **O** | **A** | **T** | **OA** | **OT** | **AT** |
|---|---|---|---|---|---|---|---|
| **O** | **0.002** | - | - | - | - | - | - |
| **A** | 0.082 | 1.000 | - | - | - | - | - |
| **T** | 0.086 | 0.848 | 1.000 | - | - | - | - |
| **OA** | 0.057 | 1.000 | 1.000 | 1.00 | - | - | - |
| **OT** | **< 0.001** | 1.000 | 1.000 | 0.424 | 1.000 | - | - |
| **AT** | **< 0.001** | 1.000 | 0.634 | **< 0.001** | 1.000 | 1.000 | - |
| **OAT** | **< 0.001** | 1.000 | 1.000 | **0.001** | 1.000 | 1.000 | 1.000 |

Pairwise comparisons using Dunn's-test with Bonferroni P-value adjustment method. Sub-categories: Normozoospermic (NO), Oligozoospermic (O), Asthenozoospermic (A), Teratozoospermic (T), Oligoasthenozoospermic (OA), Oligoteratozoospermic (OT), Asthenoteratozoospermic (AT), Oligoasthenoteratozoospermic (OAT). Statistically significant results are highlighted in bold.

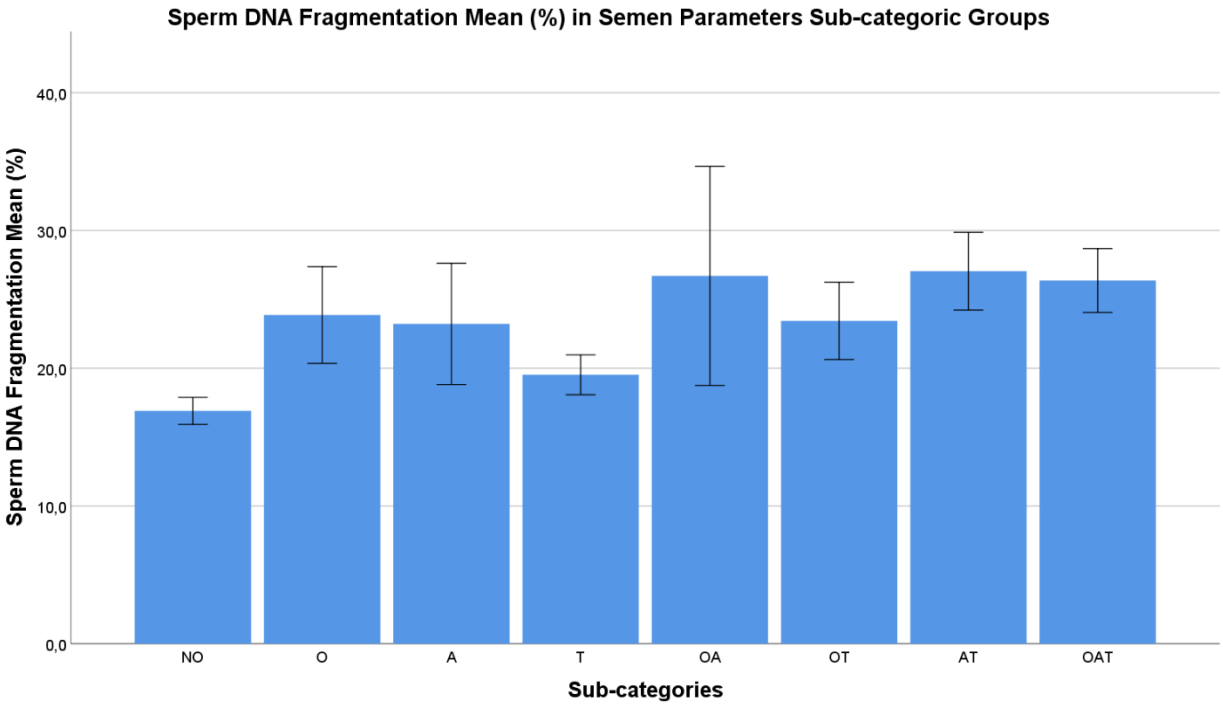


**Figure 3.** Representation of sperm DNA fragmentation mean (%) for each semen parameters sub-categoric groups with its corresponding error bars with a 95% confidence interval.

The influence of total sperm aneuploidy in the different sub-categories was also investigated. The NO group showed statistically significant differences comparing with the OT (P = 0.011) and OAT (P < 0.001) groups. Also, the T group had statistically significant P-values when compared with OT (P = 0.026) and OAT (P = 0.003) groups and the A group had statistically significant differences when compared with the OAT group (P = 0.045) (Table 8). Observing Figure 4 and comparing means of total aneuploidy, both NO (0.65 ± 0.365) and T (0.64 ± 0.333) groups had lower percentages, when comparing with the OT and OAT groups (1.10 ± 0.744 and 1.83 ± 2.716, respectively). Additionally, the OAT group had a higher percentage, when compared with the A group (1.83 ± 2.716 vs 0.82 ± 1.077, respectively).

**Table 8.** Comparisons of Total Sperm Aneuploidy between groups taking into account semen parameters.

| | NO | O | A | T | OA | OT | AT |
|---|---|---|---|---|---|---|---|
| **O** | 0.311 | - | - | - | - | - | - |
| **A** | 1.000 | 0.460 | - | - | - | - | - |
| **T** | 1.000 | 0.346 | 1.000 | - | - | - | - |
| **OA** | 1.000 | 1.000 | 1.000 | 1.000 | - | - | - |
| **OT** | **0.011** | 1.000 | 0.106 | **0.026** | 1.000 | - | - |
| **AT** | 1.000 | 0.530 | 1.000 | 1.000 | 1.000 | 0.126 | - |
| **OAT** | **< 0.001** | 1.000 | **0.045** | **0.003** | 1.000 | 1.000 | 0.053 |

Pairwise comparisons using Dunn's-test with Bonferroni P-value adjustment method. Sub-categories: Normozoospermic (NO), Oligozoospermic (O), Asthenozoospermic (A), Teratozoospermic (T), Oligoasthenozoospermic (OA), Oligoteratozoospermic (OT), Asthenoteratozoospermic (AT), Oligoasthenoteratozoospermic (OAT). Statistically significant results are highlighted in bold.

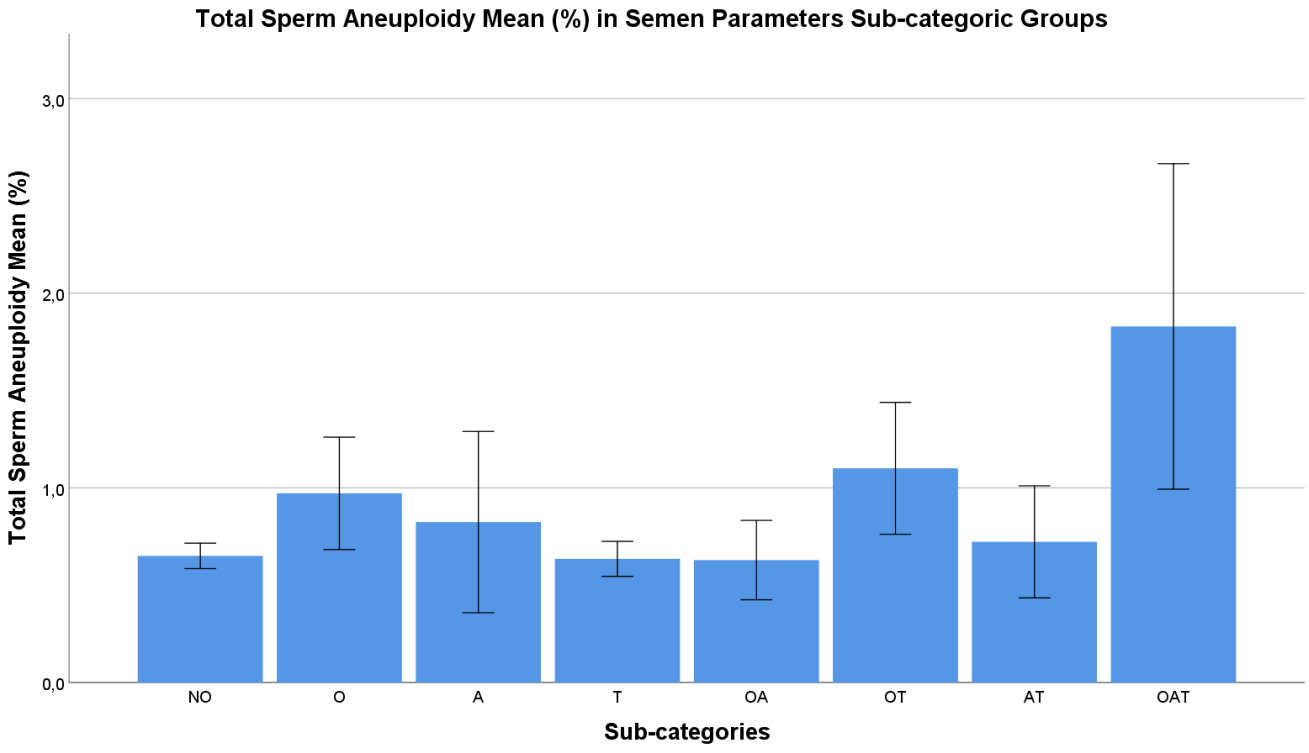


**Figure 4.** Representation of the percentage of Total Sperm Aneuploidy mean (%) for each semen parameters sub-categoric groups with its corresponding error bars with a 95% confidence interval.

Comparisons between groups considering total sperm disomy values showed only significant differences between OAT and NO (P = 0.007) and the T (P = 0.040) groups (Table 9). Figure 5 shows that the OAT group have higher percentage of total disomy (0.80 ± 0.888), when compared to NO and T groups (0.37 ± 0.225 and 0.37 ± 0.234, respectively).

**Table 9.** Comparisons of Total Sperm Disomy between groups taking into account semen parameters.

| | **NO** | **O** | **A** | **T** | **OA** | **OT** | **AT** |
|---|---|---|---|---|---|---|---|
| **O** | 1.000 | - | - | - | - | - | - |
| **A** | 1.000 | 1.000 | - | - | - | - | - |
| **T** | 1.000 | 1.000 | 1.000 | - | - | - | - |
| **OA** | 1.000 | 1.000 | 1.000 | 1.000 | - | - | - |
| **OT** | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - | - |
| **AT** | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | - |
| **OAT** | **0.007** | 1.000 | 0.106 | **0.040** | 1.000 | 1.000 | 0.361 |

Pairwise comparisons using Dunn's-test with Bonferroni P-value adjustment method. Sub-categories: Normozoospermic (NO), Oligozoospermic (O), Asthenozoospermic (A), Teratozoospermic (T), Oligoasthenozoospermic (OA), Oligoteratozoospermic (OT), Asthenoteratozoospermic (AT), Oligoasthenoteratozoospermic (OAT). Statistically significant results are highlighted in bold.

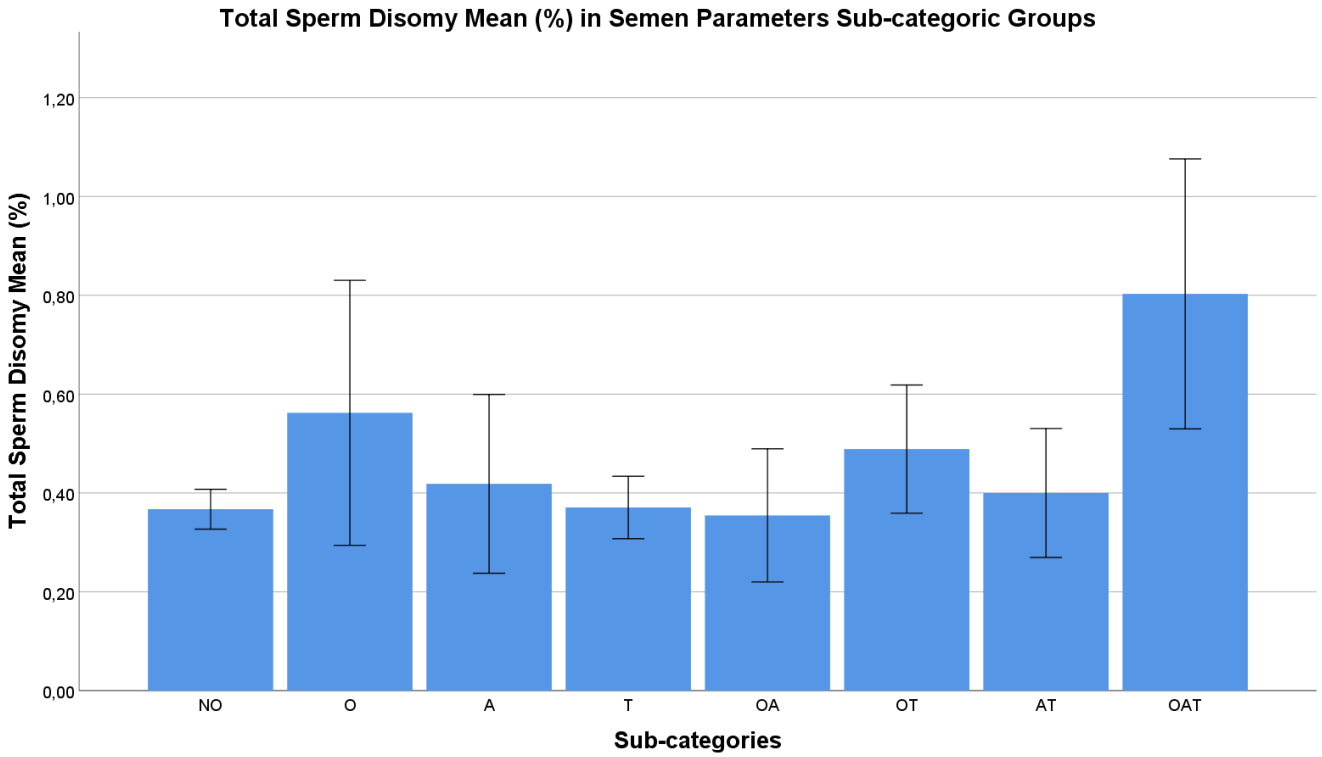


**Figure 5.** Representation of the percentage of Total Sperm Disomy mean (%) for each semen parameters sub-categoric groups with its corresponding error bars with a 95% confidence interval.

The comparisons between these groups for the presence of disomies 13, 21, 18, XX, XY, YY individually were performed but none showed statistically significant P-values.

## 4.5. Sperm DNA fragmentation *vs* Sperm aneuploidies

This study also aimed to find a correlation between sperm DNA fragmentation and sperm aneuploidies. A total of 277 patients that performed both tests were selected. A statistically positive significant correlation with sperm DNA fragmentation was found not only with sperm aneuploidies ($\rho$ = 0.269, P < 0.001) but also with the percentage of total sperm disomy ($\rho$ = 0.284, P < 0.001) and with singular disomy 13, 18 and XY ($\rho$ = 0.154, P = 0.01; $\rho$ = 0.143, P = 0.017; $\rho$ = 0.134, P = 0.025, respectively) (Figure 6). Associations with disomy 21 (P = 0.162), XX (P = 0.09) and YY (P = 0.081) were not found.

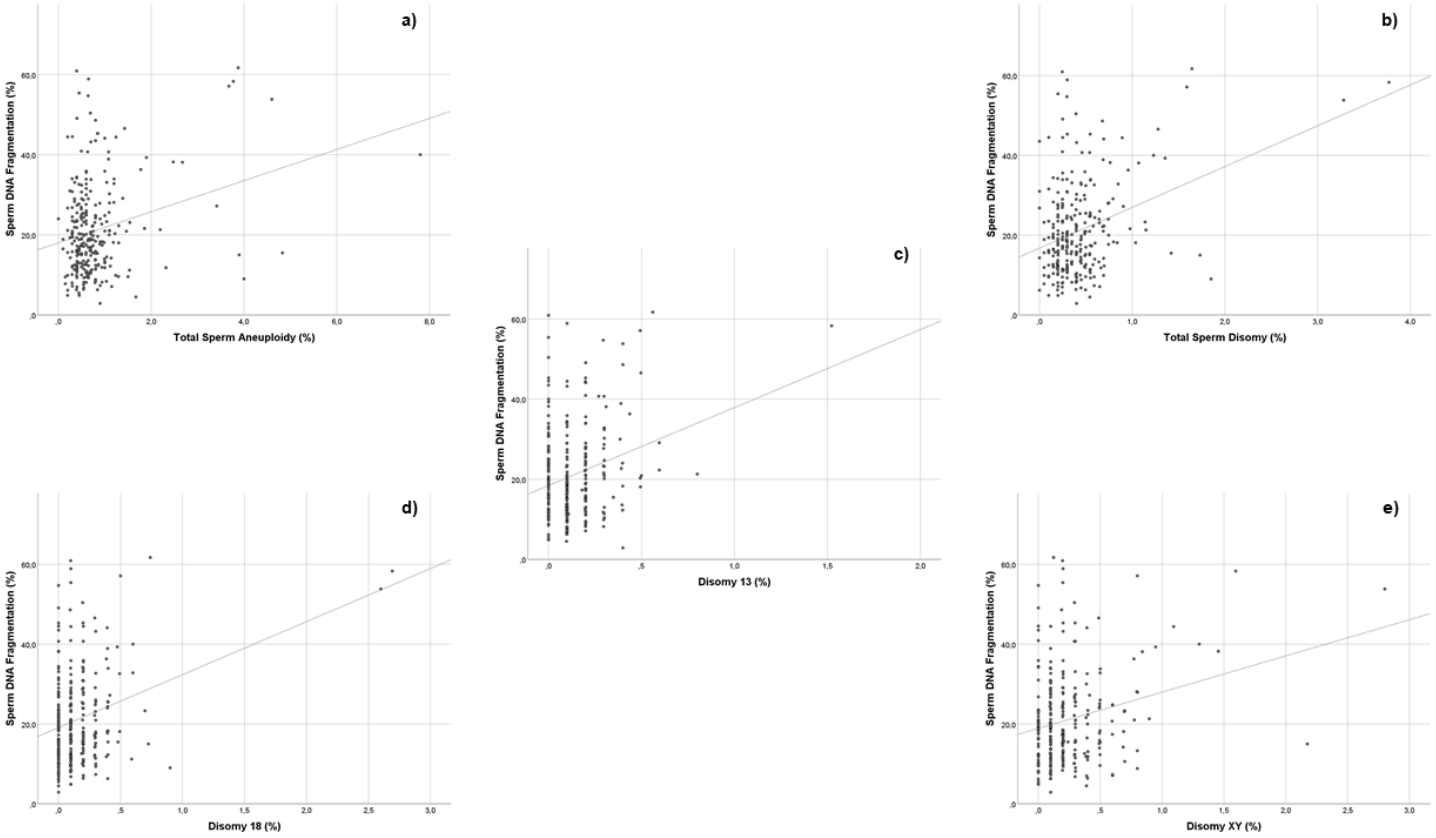


**Figure 6.** Correlations between the percentage of Sperm DNA fragmentation and a) Total Sperm Aneuploidy ($\rho$ = 0.269, P < 0.001), b) Total Sperm Disomy ($\rho$ = 0.284, P < 0.001), c) Disomy of chromosome 13 ($\rho$ = 0.154, P = 0.01), d) Disomy of chromosome 18 ($\rho$ = 0.143, P = 0.017) and e) Disomy XY ($\rho$ = 0.134, P = 0.025).

## 4.6. Defining a cut-off value for the percentage of sperm DNA fragmentation, using the TUNEL assay

The present study also aimed to define a more accurate cut-off value to our population. For this, the infertile group was compared to a group of 60 men from our NO group with secondary infertility. Results from the ROC curve and maximization of sensitivity and specificity using Youden Index, allowed to estimate a cut-off value of 18.8% for the TUNEL-assay, with a sensitivity of 53.9% and a specificity of 76.7%. The area under the curve was 0.658 with a standard error of 0.032 and P < 0.001 (Figure 7).

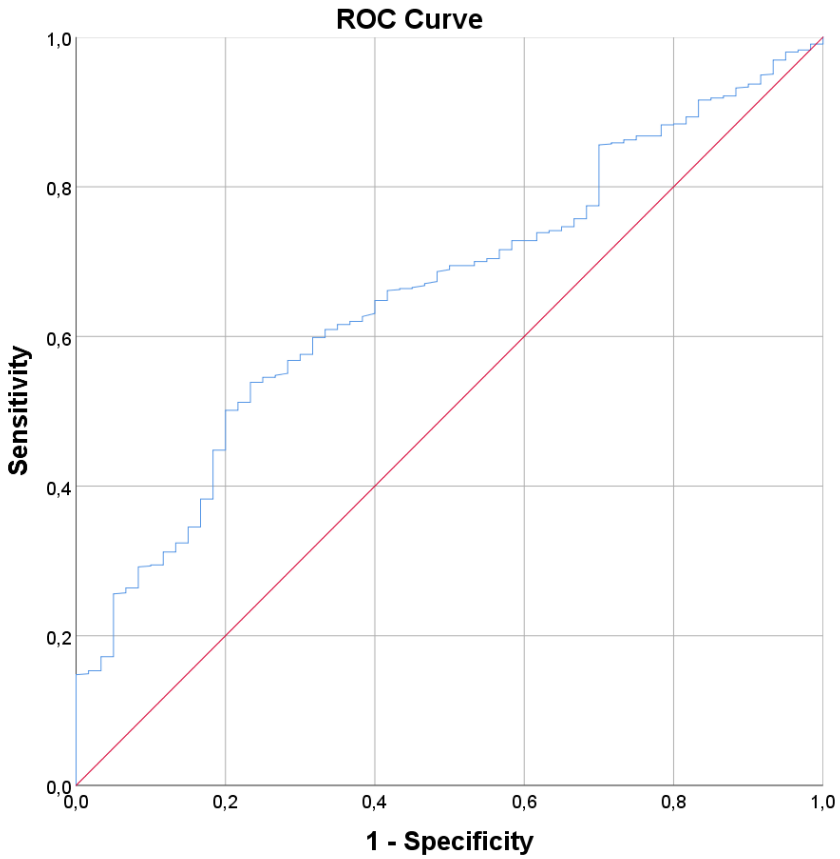


**Figure 7.** ROC curve of sperm DNA fragmentation.

# V. Discussion

Male infertility is a clinical issue with increasing interest. However, comparing with female, research, diagnosis and treatment of male infertility has been neglected, once male infertility research methods are mainly based on semen analysis which remains essentially the same from the past decades [105]. With a large sample size as ours, comparisons between male factors should be relevant, either supporting or not the literature and also to understand how these factors correlate in our demographic region.

## 5.1. The male age relevance in the infertile male population

Once couples are delaying childbearing, age became an important factor when studying couples with clinical indication of infertility.

In the literature, results obtained by different authors seem controversial. Many investigators tried to find an association between male age and semen parameters. While some did not find any relation with any parameter [26, 27], others found associations with one or more parameters [28-34, 36, 46, 47, 81]. In a retrospective study of almost 72000 infertile men in China, associations between age and some semen parameters were found. Although, for assisted reproductive technology (ART), the sperm concentration is believed to be the best predictor factor in terms of fertility [35]. With ages ranging from 24 to 76, *Brahem et al.* also showed a statistically significance between male age and sperm concentration, but not with total progressive motility or normal morphology [28]. In our study, a statistically significant correlation with sperm concentration was also observed.

Vitality also showed a statistically significant association with male age. Similar results were obtained not only by *Verón et al.*, where vitality was negatively associated with male age in a population of 11706 men [34], but also by other research groups [28, 31, 106].

Similar to total progressive motility and morphology, no significant differences were found between total chromosome aneuploidies and increasing male age in our study. Despite that, an association between male age and specifically disomies of chromosomes 13 and X was found. In spite of some studies not showing the effect of male age in sperm aneuploidy [81, 82], others found a significant increase of the diploidy

rate with male age in an infertile population [28] or proved its significant influence in sperm aneuploidy [26, 83, 84]. However, it should be weighted that these studies differ between investigations, once chromosomes included in the studies were not always the same. For example, *Cheung et al.* found a statistically significant increase of total aneuploidy rate with male age, using probes that targeted chromosomes 13, 15, 16, 17, 18, 21, 22, X and Y [84].

Male age was also compared with sperm DNA fragmentation. According to our results, age has an impact in sperm DNA fragmentation, once a statistically significant positive association was found between these two variables. Although a minor group of studies did not find a relationship between sperm DNA fragmentation and male age [28, 51], the majority found a positive association [26, 29, 31, 32, 47, 54, 106, 107], similarly to our study. In fact, our results are in concordance with *Rosiak-Gill et al.*, although they additionally found a higher incidence of sperm DNA damage in more than 40 years old men groups [46]. *Vagnini et al.* also showed an increase percentage of sperm DNA fragmentation with age, but being the threshold age value at 35 years old [45]. All these studies suggest that sperm DNA damage can be triggered by male age, having this non-modifiable factor an important influence on sperm DNA fragmentation. The causes of this damage with aging in sperm DNA can be multifactor and it has not been fully clarified [107]. Nonetheless, it is known that our organism has a preventive antioxidant system, in which antioxidant enzymes have the ability to stop an oxidation chain. A balance between them and free radicals is important for testicular function [108]. With this, failures in this antioxidant system make men more prone to oxidative attack and an excessive presence of ROS begins to accumulate, being DNA strands more susceptible to fragmentation [109]. Taking this into account, *Syntin et al.* conducted a study in rats to evaluate the expression of stress response genes in Leydig cells. The results of this study showed a decrease ratio of antioxidants with age, leading to a significant increased damage in the testicular tissue due to oxidative stress [110]. Indeed, an excessive generation of ROS has been studied and correlated with age [111] and oxidative stress and errors during spermatogenesis are more likely to occur in older men [48]. It has been also reported in several studies that increased levels of ROS are related with increased sperm DNA fragmentation, suggesting that this damage in sperm is mainly due to the high concentration of ROS, diminishing male fertility status [49, 50, 112]. Additionally, there are reports that showed a significant decrease of sperm DNA damage after antioxidant treatments [113, 114]. Not only antioxidant therapy has improved semen quality of patients with high percentages of DNA fragmentation, but also seemed to improve ICSI outcomes [115].

Thus, when male age seems to be a relevant factor, a sperm DNA fragmentation test is recommended. Additionally, and if in the presence of increased percentages of sperm DNA fragmentation, posterior treatment with antioxidants may be relevant in the success of the fertility treatments in the elder group.

## 5.2. Semen analysis, sperm aneuploidies and sperm DNA fragmentation

As previous referred, male infertility can have different causes ranging from hormonal to genetic, and not always semen analysis can provide enough information to clarify male fertility status. Thus, correlations between semen parameters, sperm DNA fragmentation and sperm aneuploidies could contribute to improve diagnosis.

In this study, it was observed that sperm DNA fragmentation had a statistically significant and negative correlation with sperm concentration, total progressive motility and morphology. Many studies have tried to find a relationship between sperm DNA fragmentation and semen parameters. Although some authors did not find a correlation between them [60], others have concluded that high levels of fragmented DNA present in the sperm are correlated with abnormal semen parameters [47, 52-55]. Additionally, statistically significant and negative correlations were found between sperm aneuploidies, sperm concentration, morphology and total progressive motility. Similar results were reported by other authors [39, 87, 91]. For example, *Vegetti et al.* studied the correlation between semen parameters and sperm aneuploidy and showed that aneuploidy rate was inversely correlated with abnormal semen parameters [97].

As associations between semen parameters, sperm DNA fragmentation and sperm aneuploidies were established, it may be relevant to investigate what sub-categoric groups of infertile men may be more indicated for sperm DNA fragmentation and/or sperm aneuploidy tests. In spite of previous work investigating sperm DNA damage and sperm aneuploidies for its relevance in the different subcategories of infertile men, individual comparisons with a control group or with a NO group were done [47, 56, 87, 91, 116-121]. In this study, we proposed to evaluate all the variables and make comparisons in a more extensive way for each subcategory group of infertile men.

First, NO and the non-normozoospermic groups were compared and statistically significant differences were observed not only in sperm DNA fragmentation, but also in total sperm aneuploidy and total sperm disomy. Other studies are in accordance with

these findings [99, 101, 122]. Considering this, the non-normozoospermic group was subdivided according to semen parameters values and comparisons between all the subgroups were performed. For that, after Bonferroni correction, Dunn's test was performed to pinpoint which specific means are significant from the others. When comparing the subcategories between them**,** sperm DNA fragmentation testing seemed more indicated for pure O individuals or for patients with association of more than one sperm abnormality (OT, AT and OAT). Although differences between OA group and any other groups were not statistically significant, for clinical counselling it may be wiser to order this test for all subcategory groups with more than one abnormal value. Moreover, significant decreased percentages of DNA fragmentation were observed in the T group when compared to the AT and OAT groups. When total sperm aneuploidy variable was studied between subgroups, the OT and OAT groups had significant differences when compared to the NO and T groups. Also, when total sperm disomy is evaluated in the different sub-categoric groups, significant differences are observed not only between the OAT group and the NO group, but also with the T group.

Analysing our results, beside the fact that more significant differences are present when comparing to the NO group, teratozoospermia isolated seems to have statistically significant lower percentages of sperm DNA fragmentation and sperm aneuploidy in comparison with the other groups (specifically to groups with teratozoospemia associated with other aberration). According to WHO 2010 guidelines, teratozoospermia is characterized by a percentage of morphological normal sperm below 4% [13]. However, this classification takes into account the head, intermediate piece, tail abnormalities and associations between them. Actually, there are investigations in this area that have differentiated the types of sperm morphology. For example, *Le et al*. reported that sperm DNA fragmentation is associated with only abnormal head. Correlations with abnormal morphology or abnormal tail-neck were not found [60]. *Tang et al.* not only correlated abnormal round sperm heads with DNA fragmentation, but also sperm aneuploidies with amorphous heads and tail abnormalities [123]. Other studies have correlated sperm DNA damage and chromosomal abnormalities with different types of abnormal morphology in the sperm [57, 106, 118], suggesting that specific types of abnormal morphology in spermatozoa can be correlated with the presence of aneuploidies and/or sperm fragmentation, although is not consensual which are more relevant. In the present study, no groups of sperm abnormal morphology were created. It was used the WHO 2010 criterion for sperm morphology classification, where abnormal morphology represents all the types of morphologic anomalies observed.

Additionally to sperm concentration, morphology and total progressive motility, sperm DNA damage with vitality and hypoosmolality associations were found. In fact, correlations between sperm DNA fragmentation with vitality and hypoosmolality revealed to be negative correlated. Although some authors did not find this correlation with viability [60], an inverse relationship between sperm DNA fragmentation, vitality and hypoosmolality have already been described [47, 54, 61, 106]. *Samplaski et al.* focused on the predictive value that vitality can have in sperm DNA fragmentation level. Not only they found a strong correlation ($\rho$ = -0.83) between these variables, but also reported that men with sperm vitality percentages higher than 75 are very likely to have low sperm DNA fragmentation levels. Similarly, when sperm vitality is below 50%, sperm DNA fragmentation may not be useful once more than 95% of these men are likely to have high levels of DNA damage. It should be stressed that this investigation was performed using SCSA and a threshold value at 30% for sperm DNA fragmentation and they concluded that analysing only the sperm vitality, sperm DNA fragmentation test should not be required once it does not provide any supplementary information. Our study focused only on the viability and sperm DNA fragmentation association and not in the levels of these parameters and its associations. In fact, DNA damage is negatively associated with viability, but not strongly enough for one test substitute the other ($\rho$ = -0.553). Interestingly, vitality correlated negatively with both male age and sperm DNA fragmentation. Moreover, as it was mentioned before, for male age and sperm DNA fragmentation a statistically significant positive correlation was found. Therefore, this suggests that these three factors are correlated and both APA and low vitality may be a good indication to request a sperm DNA fragmentation test.

## 5.3. Sperm DNA fragmentation vs Sperm aneuploidies

As sperm DNA fragmentation and sperm aneuploidies could be relevant for male infertility, it might be worth to evaluate if there is also an association between these two variables. In fact, previous works have been reported addressing this issue. One study performed by *Muriel et al.* in 16 men evaluated the correlation between sperm aneuploidy rates and sperm DNA fragmentation in the same sperm cells. Results have reported that an increased rate of DNA fragmented was found in sperm cells with sex chromosomes aneuploidies, although aneuploidy was only determined for chromosomes 18, X and Y [100]. According to *Enciso et al.*, a significant correlation between sperm DNA damage and sperm aneuploidy was also observed, with aneuploidies measured for

chromosomes 13, 16, 18, 21, 22, X and Y in a 45 infertile male population [102]. One study questioned whether DNA damage was more frequent in sperm cells with the presence of the Y or the X chromosome. Results showed that sperm cells are more vulnerable to DNA fragmentation when Y chromosome is present [101]. However, there are studies that did not find any correlation. *Balasuriya et al.* performed a test where simultaneous detection of aneuploidy and DNA fragmentation of the same sperm cells was possible, using SCD-fluorescent *in situ* hybridization. With a study population of 20 males, no significant association between these variables was found [103]. Another study also aimed to investigate the relationship between these factors in 38 infertile men and similar results were reported [104]. These non-consensual studies might occur due to the different number of chromosomes analysed, different methods used to evaluate the sperm DNA fragmentation index and even due to different protocols of semen preparation. So, in order to have valid information, it is important to compare results from studies with similar populations and methods used.

In our study, TUNEL-assay and FISH technique were both performed in a 277 patients' population. A statistically significant positive correlation was found between sperm DNA fragmentation and total sperm aneuploidy, total sperm disomy disomies for chromosomes 13, 18 and XY, individually. Using the same approach, similar results were found by other researchers [98, 99]. *Arumugam et al.* conducted a study to find an association between sperm aneuploidy and sperm DNA fragmentation in infertile men, measuring specifically aneuploidy rates for chromosomes 13, 18, 21, X and Y. Their results were similar to ours, being necessary to mention that their population was composed only by 100 infertile men, being 68% of them azoospermic [98]. *Di Santo et al.* also performed a comparable study using these two techniques and the same chromosomes studied. In this case, a 109 infertile patient population with abnormal semen parameters was studied and a significant positive correlation was also found. However, it is important to stress that semen preparation for the TUNEL-assay had a double gradient centrifugation, where spermatozoa are selected and thus lowering the sperm DNA fragmentation percentage levels [99].

After analysing all these studies previously mentioned and including our own results it might be conclude that sperm aneuploidies and sperm DNA fragmentation are positively correlated. Many explanations have been proposed for this association, but probably the most consensual hypothesis relays on the escape of abnormal sperm cells from apoptosis. To form a mature spermatozoon, diploid cells undergo through mitosis since fetal live and suffer meiosis when arriving puberty. During these cell divisions, cellular control occurs and checkpoints are crucial to maintain the normal formation of a

mature sperm cell. With this, the proliferation of abnormal cells with meiotic errors can be blocked and induction of apoptosis in these cells can occur [124]. However, some cells seem to escape this programmed cell death, even when they were marked for apoptosis. This abortive apoptosis appears to be linked to the existence of abnormal spermatozoa in the ejaculate [125]. Inside of the mechanisms that could induce cell death, DNA fragmentation has been proposed as one of the mechanisms designed to inactivate abnormal sperm [100]. By escaping apoptosis, aneuploid sperm cells with fragmented DNA can be present in the ejaculated sperm and be an indicator of a spermatozoon that was marked for apoptosis and escaped it [102, 126]. In fact, a study performed by *Enciso et al.* sustained this hypothesis by the evaluation of both parameters in the same sperm cell. Indeed, by analyzing individual male gametes, they found sperm damage and sperm aneuploidies present in the same sperm cells [102]. However, the presence of DNA fragmentation in sperm does not mean that sperm aneuploidies are also present, as sperm damage does not only occur when cells are marked for apoptosis. Indeed, sperm damage can happen due to other internal and external factors [43, 126]. Nevertheless, a high percentage of DNA fragmentation can be indicative of chromosomal abnormalities and a detailed investigation should be performed to avoid the conception of embryos with an abnormal chromosome complement. Therefore, more detailed investigations and evaluation of risks should be performed.

## 5.4. Defining a cut-off value for fluorescence microscopy TUNEL

For a better clinical interpretation, it is important to know which technique is used to study sperm DNA fragmentation, as different methods require different threshold values. Moreover, a consensus does not exist for the percentage of sperm DNA fragmentation above which the reproductive capacity is compromised. Even using TUNEL-assay, different cut-off values have to be postulated, whether measurement of sperm fragmentation is performed with flow cytometry or fluorescence microscopy [66]. Additionally, only a few studies attempted to define a cut-off value for TUNEL-assay by fluorescence microscopy. For example, *Henkel et al.* have proposed 36.5% as a cut-off value (with 75% sensitivity and 43.1% specificity) for TUNEL with an epifluorescence microscope, although the discriminator factor was the success of pregnancy (N = 167) [72]. More recently, *Javed et al.* investigated the predictive value of TUNEL-assay in

male infertility, among other techniques. For the TUNEL-assay, 95 patients were included and a cut-off value was defined at 22.08% with 75.4% sensitivity and 94.2% specificity [71]. Threshold values were also defined by *Henkel et al.*, but different cut-offs were proposed for different group of treatments. In a population of 208 individuals that went through IVF, TUNEL cut-off value was defined at 36.5%, with 80.6% sensitivity and 34.9% specificity, where pregnancy rates were significantly lower for the TUNEL-positive spermatozoa. On the other hand, a threshold at 24.3% was obtained for the distinction of TUNEL sperm results in the ICSI group, with 66.7% sensitivity and 63.6% specificity, in a 54 male population [73]. However, these cut-off values were specific for IVF and ICSI treatment groups. In our study, sperm DNA fragmentation cut-off percentage obtained for TUNEL-assay with fluorescence microscopy was lower. Analysing and maximizing both sensitivity and specificity, a cut-off value of 18.8% was achieved with 53.9% sensitivity and 76.7% specificity.

As far as is our knowledge, this was the first study performed in order to define a threshold value for the TUNEL-assay using fluorescence microscopy, in our demographic region and using such a high sample (N = 809). Therefore, we recommend that the cut-off of 18.8% for sperm DNA fragmentation should be used as the reference value above which male fertility status could be seriously compromised. Considering this, a better reproductive counselling may be offered to couples.

# VI. Conclusion

The study of male infertility plays an important role in the investigation for a couple infertility problem. Finding the associations between male infertility factors in our demographic region could improve the reproductive outcomes in our population. However, defining the fertility status of an individual based only on semen parameters will give poor information, as sperm DNA status is not provided.

Our results support that men with APA should be indicated for sperm DNA fragmentation testing along with a vitality test.

Semen parameters were shown to be negatively correlated with sperm DNA fragmentation and sperm aneuploidies. Sperm DNA fragmentation testing might be more appropriate for men with only oligozoospermia or with other semen abnormalities in association, whereas sperm aneuploidy testing might be more indicated for OT or OAT men. In addition, sperm DNA fragmentation and sperm aneuploidies tests can give complement information about the fertility status and therefore should be included in male infertility diagnosis routine investigation.

For the first time, and to the best of our knowledge, a threshold value for the TUNEL-assay by fluorescence microscopy was established at 18.8%. This new information will provide a better patient classification and, consequently, a better reproductive counselling, hopefully increasing rates of fertility treatments well succeeded.

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