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RESEARCH & LABORATORY INTERNSHIP IN ANDROLOGY/MALE INFERTILITY

VOLUME 1

Internship report submitted in partial fulfilment of the requirements for the award of Master's degree (MSc) in Laboratory Clinical Genetics, under the supervision of Dr. Rosário Pinto-Leite, PhD and Professor Dr. Ilda Ribeiro, PhD, and presented to the Faculty of Medicine, University of Coimbra.

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RESEARCH & LABORATORY INTERNSHIP IN ANDROLOGY/MALE INFERTILITY

Effects of Cryopreservation on Spermatic Parameters, DNA Integrity, and Mitochondrial Activity

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ACKNOWLEDGEMENTS

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ABSTRACT

As the central focus of andrology, male reproductive health deals with various topics, from infertility, male contraception, and erectile dysfunction to male senescence. The present internship report addresses two key aspects that currently challenge male fertility studies: male fertility analysis (Section A) and preservation (Section B).

Section A follows the development of a retrospective cross-sectional study in which the populational traits of men attending fertility consultations and semen donors alike were subjected to analysis. Consequently, the results of the semen analysis, cytogenetic and molecular studies of more than 1,000 men were reviewed. The incidence of cytogenetic abnormalities in infertile male patients residing in Trás-os-Montes and Alto Douro was 2.6%, with no detection of microdeletion of the Y chromosome. Lastly, male fertility evaluations at the Trás-os-Montes and Alto Douro Hospital Centre (CHTMAD) were mainly composed of overweight men over 35 years of age who suffer from primary infertility with lifestyle habits that closely resembled those reported for the (male) Portuguese population in general.

Section B describes a pilot study on the effect of the antioxidants astaxanthin (ASTX) and vitamin E (VE) on sperm cryopreservation. Rapid freezing cryopreservation was performed on six normozoospermic semen samples, divided into three main groups of 0, 2.5 and 5.0% (v/v) dimethylsulfoxide (DMSO). Furthermore, the last two groups had their freezing medium supplemented with different concentrations of ASTX and VE (0, 15, 25 μM of ASTX, 5 mM VE and 25 μM ASTX + 5 mM VE) in their respective DMSO concentration (v/v). For each group, washed semen aliquots were used, with a concentration adjusted to 20 million/ml sperm cells. Thawing was performed after two weeks. Sperm motility, vitality (Eosin Y 0,5%), morphology, and DNA fragmentation (Alkaline Comet-Assay) were evaluated before freezing and after thawing. VE was the antioxidant with the poorest outcome for sperm total motility (10%) and vitality (14%). Five per cent DMSO was generally more beneficial for all the studied parameters, with significant improvement in sperm vitality (40.8%) and DNA damage (22.2%), over the control group. Moreover, ASTX at 5.0% DMSO displayed the most marked impact on DNA damage. The concentrations of 15 and 25 μM resulted in minor DNA damage (29.8% and 34.3%, respectively). In comparison, the control group exhibited significantly higher values (60.8%). Furthermore, our findings suggest that 5.0% DMSO in combination with ASTX shows promise in improving sperm vitality and reducing DNA damage. However, finding the right balance between these two substances is crucial to avoid toxic effects. Understanding the interplay between DMSO, ASTX, and their concentrations can be essential for effective sperm cryopreservation strategies and thus merits further investigation.

In summary, the research and laboratory internship in andrology/male infertility provided practical exposure to laboratory work relevant to andrology and male fertility, an opportunity to participate in research projects and generate knowledge. The latter was accomplished through the publishing of research findings and presentations at conferences.

Keywords: Andrology, Male Infertility, Semen Analysis, Cryopreservation; Antioxidants

PUBLICATIONS

Partial results of the presented work have been published in the form of conference proceedings (abstracts/oral communications, and posters) or articles:

Li, F., Arantes, R., Gaivão, I., Moutinho, O., Pinto-Leite, R. (2023). Astaxanthin Shields Sperm DNA Against Freeze Damage: A Preliminary Study. Austin Journal of Reproductive Medicine & Infertility (under review). **Appendix I. ORIGINAL ARTICLE.**

Li, F., Arantes, R., Souto, M., Pinto, C., Matos, A., Gomes, Z., Moutinho, O., Pinto-Leite, R. (2023). Cytogenetic Findings in Infertile Couples from Trás-os-Montes and Alto Douro Region: A Glimpse into The Genetic Basis of Infertility. Proceedings of the 26th Annual Meeting of the Portuguese Society of Human Genetics (SPGH – Sociedade Portuguesa de Genética Humana), 102(13), e33154. https://doi.org/10.1097/MD.0000000000033154. **Appendix II. ABSTRACT & POSTER SPGH 2023.**

Li, F., Arantes, R., Ribeiro, I.P., Moutinho, O., Pinto-Leite, R. (2022). The Challenge of Cryopreservation for Male Fertility Preservation. XIV Jornadas de Bioquímica, Vila Real, Portugal.**Appendix III. ORAL COMMUNICATION – XIV JORNADAS DE BIOQUÍMICA.**

Li, F., Arantes, R., Moutinho, O., Pinto-Leite, R. (2022). The Breaking Point: Impact of Reciprocal Translocations on Male Infertility. XIV Genetics and Biotechnology Conference / IV Genetics and Biotechnology Iberian Conference, Vila Real, Portugal. **Appendix IV. POSTER – XIV GENETICS AND BIOTECHNOLOGY CONFERENCE.**

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A	Asthenozoospermia
ACA	Alkaline Comet-Assay
APN	Portuguese Association of Nutritionists
ART	Assisted Reproductive Techniques
ASTX	Astaxanthin
AZ	Azoospermia
AZF	Azoospermia Factor
BMI	Body Max Index
BTB	Blood-Testes-Barrier
C	Criptozoospermia
CBL	C-Bands by Barium Hydroxide, Using Leishman
CHTMAD	Trás-Os-Montes and Alto Douro Hospital Centre
CNV	Copy Number Variation
Conc.	Concentration
COVID-19	Coronavirus Disease 2019
CPA	Cryoprotective Agent
Ct	Threshold Cycle
DHT	Dihydrotestosterone
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediamine Tetraacetate
EG	Ethylene Glycol
EPE	Public Business Entity
FBS	Foetal Bovine Serum
FISH	Fluorescent In Situ Hybridisation
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
GTL	G-Bands by Trypsin Using Leishman
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HPG	Hypothalamic-Pituitary-Gonadal
ICMART	International Committee for Monitoring Assisted Reproductive Technology
ICSI	Intracytoplasmatic Sperm Injection
IIF	Intracellular Ice Formation
IM	Immotile
IUI	Intrauterine Insemination
IVF	In Vitro Fertilization
LH	Luteinizing Hormone
MTX	Methotrexate

N	Normozoospermia
NGS	Next Generation Sequencing
NIOSH	National Institute for Occupational Safety and Health
NP	Nonprogressive Motility
O	Oligozoospermia
OA	Oligoasthenozoospermia
OAT	Oligoasthenoteratozoospermia
OT	Oligoteratozoospermia
PR	Progressive MOTILITY
PROH	1,2 Propanediol
qPCR	Real-Time Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RPL	Recurrent Pregnancy Loss
RT	Room Temperature
SAT	Sperm Aneuploidy Test
sDF	Sperm DNA Fragmentation
SFM	Sperm Freezing Medium
SNP	Single Nucleotide Polymorphism
SPM	Sperm Preparation Medium
SRY	Sex-Determining Region Y
STS	Sequence-Tagged Site
T	Teratozoospermia
TMSC	Total Motile Sperm Count
TNSC	Total Normal Sperm Count
TPMSC	Total Progressive Motile Sperm Count
TSC	Total Sperm Count
TZI	Teratozoospermia Index
VE	Vitamin E
VNTR	Variable Number Tandem Repeat
WHO	World Health Organisation
YCMD	Y Chromosome Microdeletions

“Nature has designed life, from the smallest microbe to the largest whale, to have one basic purpose. That purpose is to reproduce. Whatever else an organism does, reproduction is its basic goal. All else, from dolphins frolicking, to the pyramids, is gravy.”

Richard F. Taflinger, in Biological Basis of Sex Appeal,

INTRODUCTORY NOTE

The English novelist Samuel Butler, faced with the chicken or the egg causality dilemma, had a thought-provoking answer: *'The hen is the way of making another egg',* which means that all biological systems exist only to perpetuate themselves [1].

For biologists, generational renewal through reproduction is an essential element of the continuity of life or an elegant mechanism of physical persistence and senescence evasion of species [2]. Reproduction is the process of creating new individuals from existing ones. The human reproductive process is nothing short of astonishing. Among a reservoir of millions of oocytes, a relatively small number are ovulated; among the millions of male sperm, one reaches the oocyte, and fertilisation occurs (a process that merits special recognition on its own!). Furthermore, 40 to 50% of conceptions are spontaneously aborted [3]. So, in any given month, couples with normal functioning reproductive systems have only a 25% chance of conceiving (despite multiple copulations), and only 70% of couples will achieve conception after six months [3]. In truth, successful reproduction requires not only a confluence of a myriad of processes and conditions but a well-orchestrated succession of events (germ cell differentiation, gametogenesis, ovulation, fertilisation, preimplantation embryo development, implantation, decidualisation, placentation, and parturition) as well [4]. Each event impacts the outcome of the one before; therefore, any impaired process along this pathway prohibits live birth.

According to the definition of the World Health Organisation (WHO) and the International Committee for Monitoring Assisted Reproductive Technology (ICMART), infertility describes the failure to achieve a clinical pregnancy after at least 12 months of regular unprotected sexual intercourse [5]. Its global prevalence ranges from 3.5% to 16.7% in developed countries and 6.9% to 9.3% in less developed [6]. Against this backdrop, a theoretical-conceptual framework is provided regarding male infertility, its numbers worldwide, its impact, and trends. However, the core volume of this manuscript illustrates all the work developed in the Hospital Centre of Trás-os-Montes and Alto Douro (CHTMAD) andrology laboratory. Every aspect of the internship is tackled one section at a time. First, a descriptive study exploring the main characteristics of men attending fertility consultation at CHTMAD: lifestyle traits, semen profile, and cytogenetic and molecular findings. Second, to shed light on the preservation of male fertility, a pilot study involving freezing medium supplementation with antioxidants was conducted.

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SECTION A. MALE FERTILITY ANALYSIS

"... the final word of evolutionary biology always seems to come to this: death is the engine of nature."

- Paul Santmire [7]

I. INTRODUCTION

Mortality, cessation, and ends are the driving forces of all that is alive [8]. Despite the plethora of reproductive modes found in nature, two broad categories, asexual and sexual, distinguish the underlying mechanisms and distinct reproductive strategies.

Sexual reproduction is a mode of reproduction that produces offspring with genomes derived from the association and/or reassortment of genetic material from more than one origin. The new genome results from the fusion of two cells, the gametes, into a single cell, the zygote. Two main processes dwell in this form of reproduction: genetic recombination through the production of gametes (with distinct and unique genomes) and syngamy, the fusion of two genomes (from two gametes) into one (the zygote). Meiosis accomplishes the first step through reductive divisions of germline cells that generate gametes with half the normal number of chromosomes (haploid cells). Then fertilisation restores the normal number of chromosomes.

1. The Male Reproductive System

Evolutionary fitness is dependent on successful reproduction. The female and male reproductive systems provide the means for the sexual maturation of each individual and the production of gametes. Despite their differences, both systems share several key characteristics [9]: homologous structures; the presence of primary sex organs, the gonads, which (besides gamete production) secrete sex hormones that affect maturation, development, and promote changes in the activity of the reproductive system organs; the presence of accessory reproductive organs, and the existence of a dormant and non-functional period until the onset of puberty. From puberty onwards, fertility and reproductive activity are continuously maintained. Cyclic variations do not occur in the levels of pituitary hormones or testosterone. Spermatogenesis, likewise, is continuous [9].

The internal and external genital organs comprise the male sex organs. The penis and scrotum represent external sexual organs. The male gonads (testis), epididymis, ducts, and accessory glands constitute the male internal genital organs (**Figure A.1**):

a) The penis is the male copulatory organ. It comprises three erectile tissues: a paired dorsal corpora cavernosa penis and a single ventral corpus cavernosum urethrae. The glans penis is supplied with sensory nerve endings and is the primary erogenous zone in the male. The expulsion of urine and semen fluid is the penis primary function. The scrotum is a cutaneous sac that covers the testes and the lower parts of the spermatic cord. Its prominent role is to ensure optimal temperature for sperm production in the testes [3, 10].

b) Testes are paired ovoid structures enclosed by a fibrous capsule (tunica albuginea). The testes are responsible for the production of sperm and hormones necessary for the development and maintenance of male sexual characteristics. Each testis is divided into 200 to 300 lobes. Each lobule contains up to four highly convoluted loops, the seminiferous tubules. These tubules are populated by germ cells and Sertoli cells. The interstitial tissue packed between the seminiferous tubules has an endocrine role (steroid-secreting cells). Sperm cells develop in the seminiferous tubules and pass into the rete testes, a system of anastomosing channels, where a mixture of products from the individual lobules occurs. The interstitial compartment occupies one-third of the total testicular volume and comprises Leydig cells, blood vessels, nerves, and macrophages [11].

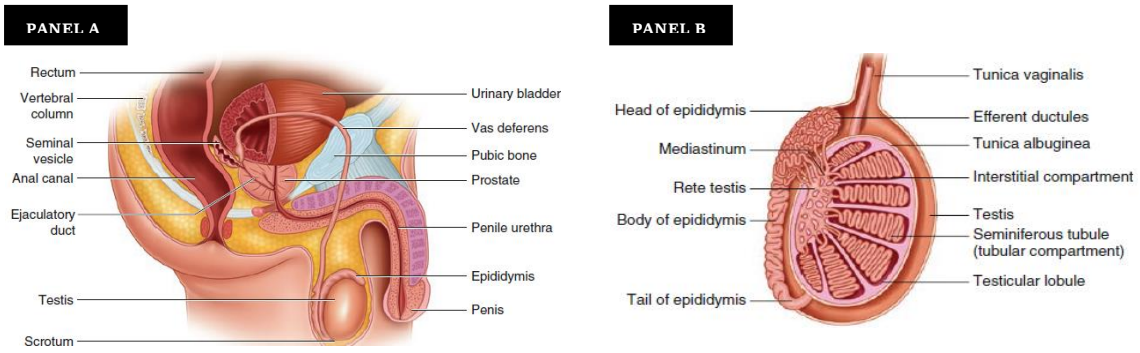


Figure A.1 - The Male Reproductive System. *Panel A – Sagittal section of the male pelvis. Panel B – Longitudinal section of testes. Adapted from Kumar & Sharma* [10].

. Sertoli cells express the *SRY* (sex-determining region Y) gene and produce the anti-Mullerian hormone. Among other functions, they help support, protect and provide nutrition for spermatogenic cells, contribute to paracrine and endocrine control of spermatogenesis [12–14], and maintain the blood-testes-barrier (BTB)[14].

. The blood-testes-barrier is a structure composed of specialised junctions between adjacent Sertoli cells close to the basement membrane of the seminiferous epithelium [15]. This structure divides the germinal epithelium into two compartments: basal and adluminal. Spermatogonial stem cells and primary spermatocytes populate the former compartment. On the other hand, the adluminal compartment is populated by secondary spermatocytes, spermatids, and mature sperm. The BTB's primary function is to restrict the passage of nutrients and steroids across Sertoli cells into the adluminal compartment [10], thus allowing the maturing spermatozoa to develop in an immunologically privileged region. This barrier also allows for the sequestering and increasing local testosterone concentrations necessary for spermatogenesis [14].

. Leydig cells are the androgen-producing cells of the testes. Leydig cell functions are regulated by luteinising hormone (LH) and thyroid hormones [11].

c) The epididymis is a mass of duct tissue that runs down and posteriorly to each testis. The organ arises from packed coiled ducts. Then, these join and form a single coiled epididymal duct where sperm maturation and storage occur [9–11].

d) The ductus deferens is composed of a thick wall of smooth muscle longitudinally lined by the mucosa of the pseudostratified epithelium that contains stereocilia (large, nonmotile microvilli). The ductus, while in the extra-abdominal section of its course, is accompanied by the testicular artery, the pampiniform plexus of veins, a nerve plexus, and bundles of skeletal muscle fibres (arising from the anterior abdominal wall) comprising the cremaster muscle. The gathering of these structures forms the spermatic cord [10].

e) Seminal vesicles are sacs that arise from each ductus deferens and can be found between the ampulla and the ejaculatory duct. The primary function of seminal vesicles is to produce seminal plasma components such as fructose, semenogelin, and prostaglandins and to help sperm travel through the male and female reproductive tracts [10].

f) The prostate is a single muscular glandular exocrine gland surrounding the initial part of the urethra. It is the largest accessory male sex gland. Changes in prostatic fluid composition or secretion affect sperm function and contribute to male infertility [11].

e) Cowper glands are paired glands located just below the prostate. They secrete mucins that are expelled before ejaculation and serve to neutralise the acidity of the residual urine in the urethra. It also has a lubricant role [10].

h) In men, the urethra is shared by both the urinary and reproductive tracts. The urethra has three distinct segments: the prostatic urethra, the short membranous urethra, and the cavernous urethra present along the length to the tip of the penis [10].

2. The Hormones in Male Reproduction

The hypothalamic-pituitary-gonadal (HPG) axis governs male reproduction, development, and maintenance of male sexual characteristics [10].

Puberty begins when the hypothalamus increases the production of Gonadotropin-releasing hormone (GnRH), further stimulating the anterior pituitary to release Follicle-stimulating hormone (FSH) and LH. As FSH and LH levels increase, gonads respond by secreting high levels of sex hormones and thus begin gamete and sexual maturation. A closed negative-loop feedback mechanism is responsible for the dynamic equilibrium of serum levels of these reproductive hormones [11].

Androgens are the prominent steroids the testes produce, and inhibins and activins are the main testicular protein hormones [10]. Synthesised from cholesterol in Leydig cells, testosterone is a sex steroid hormone. It plays a vital role in developing testes and accessory glands and promotes secondary sexual characteristics. Testosterone is also responsible for muscle mass, bone mass, and body hair growth [16]. Approximately 40% of testosterone is bound to sex hormone-binding globulin, and 2% is found in free form. In contrast, the remaining is weakly bound to albumin and can be quickly recruited to target tissues [10, 16, 17]. In the brain or peripheral tissues, testosterone is metabolised to dihydrotestosterone (DHT) or estradiol. DHT is responsible for developing external genitalia, accessory glands, and secondary sexual hair. Estradiol plays a role in brain sexual differentiation, bone mass accretion, and the fusion of epiphyses [16].

Inhibin and activin inhibit or stimulate FSH production, respectively. Inhibin positively correlates with Sertoli cell number, sperm concentration, and spermatogonial status, thus assuming a biomarker role for testicular function [10].

3. The Sperm Cell

A dramatic distinction between male and female gametes across metazoan exists. Whereas the oocyte is large, immotile, and with a spherical nucleus, the sperm are small, highly motile, and with various shapes. Sperm is a specialised cell formed by the cell process of meiosis and maturation. Its structural components arise due to intense selection pressures on their form and function. As such, they are the most morphologically diverse animal cell type [18].

3.1 The Structure

Human sperm is about 50-60 μm long, with a flagellum that spans 90% of its length [19] (**Figure A.2**). Sperm undergo various structural modifications in the testes, epididymis, and while travelling through the female reproductive tract. All these modifications allow the sperm not only to survive in the female reproductive tract but also to reach the egg vestments and penetrate and fertilise the egg [20].

3.1.1 The Head

The head is approximately 4 μm long and consists of a nucleus, an acrosome, and a cytoskeleton. The nucleus is in a highly condensed state. Protamines, small and basic proteins rich in arginine and cysteine, replace histones [10, 20]. Through the formation of disulphide bonds with adjacent DNA strands, sperm nucleus hyper condensation is achieved. The acrosome is an organelle derived from the Golgi apparatus. It consists of a double membranous cap found in the anterior part of the head and contains enzymes for penetration of egg vestments (cumulus oophorus, zona pellucida, oocyte plasma membrane) [10, 20].

3.1.2 The Flagellum

The basic structure of the flagellum is the axoneme. Outer dense fibers and fibrous sheath surround the latter. Outer dense fibers are present in the midpiece (enveloped by a mitochondrial sheath) and the principal piece [10].

3.2 Spermatogenesis

Spermatogenesis is a complex cellular event that describes the process of male gametogenesis, from the primordial germ cell to the sperm. In humans, spermatogenesis takes an average of 74 days [21]. It features three stages: the mitotic proliferation of spermatogonial stem cells, meiotic division and spermiogenesis [22].

The efficiency of spermatogenesis varies by species. In men, the daily rate of spermatozoa production is 3-4 million per gramme of testicular tissue [23]. However, only 12% of the spermatogenetic potential is available for reproduction [24]. Furthermore, with age, daily sperm production declines: loss of Sertoli cells, increase in germ cell degeneration, reduction in Leydig cells, myoid cells, and loss of primary spermatocytes are hinted as probable causes for this observation [22].

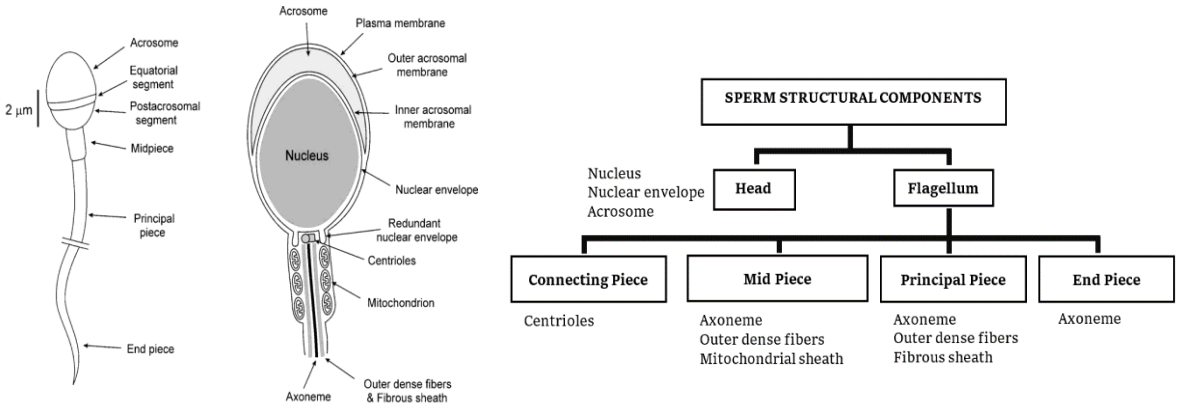


Figure A.2. Diagram and structural components of the sperm cell. *Adapted from Darszon et al.* [19] *and Kumar & Sharma* [10].

Humans and, by extension, all primates, have, regarding their differentiation state, two types of spermatogonial cells: type A and B. Type A spermatogonia are the actual stem cells of the testes: they renew their population and differentiate to give rise to type B spermatogonia [22, 25]. The latter, in turn, divide mitotically to form primary spermatocytes, secondary spermatocytes, and spermatids [26]. Even after meiosis, spermatogonia remain joined by intercellular bridges. These structures allow for biochemical interactions and synchronisation of germ cell maturation [26, 27].

Following the formation of spermatids, no further division occurs. Instead, these cells undergo a series of changes that transform them into sperm through a process termed

spermiogenesis. Events associated with this process include nuclear modifications, acrosome formation, and tail structure formation [22]. As spermiogenesis progresses, mitochondria assemble in a spiral around the proximal region of the flagellum; the remainder of the cytoplasm (residual body) moves away from the nucleus and eventually is shed along the developing tail. Sertoli cells are then responsible for the phagocytosis of the residual bodies [10].

3.3 Sperm Transport and Maturation

Sperm cells travel through both the male and female reproductive tracts. This transit serves a structural and functional maturation purpose in the male tract.

Following spermiogenesis, spermatozoa are morphologically mature but nonmotile and incapable of oocyte fertilisation. Through the rete testes and the efferent ducts, sperm cells reach the epididymis caput. There, the sperm cells must journey ten days to 2 weeks, toward the epididymis cauda, for complete maturation. During this time, the epididymis secretes proteins that lead to the modification of sperm surface proteins, changes in the plasma membrane, and incorporation of proteins into the cellular components of sperm [10]. Upon ejaculation, spermatozoa move through the ductus deferens and mix with fluid secretions from the male accessory glands. The seminal fluid is deposited in the upper vagina, where its composition and buffering capacity shield the sperm from the vaginal acid environment [10].

The penetration of cervical mucus is the next barrier to be overcome. The cervical mucus's viscosity and composition vary considerably throughout the menstrual cycle. Around the time of ovulation, the production of watery cervical mucus (E mucus) facilitates the movement of the sperm through the cervix; after ovulation, a sticky mucus with low water content, progestational, or G mucus, is now present, making it almost impossible for the sperm to penetrate such mucus. In the uterine cavity, uterine smooth muscle contraction is the primary transport mechanism [28]. Once in the isthmus, sperm bind to the epithelium for approximately 24 hours and undergo capacitation: a prerequisite for hyperactivated motility and the acrosome reaction. Through a mixture of the tube's muscular movements and the swimming motion of the sperm cells, the spermatozoa progress towards the oocyte. Peristaltic contractions of the uterine tube simultaneously transport the oocyte down the tube and the sperm up the tube. Oocyte fertilisation occurs typically in the ampullary portion of uterine tubes [3, 10, 28].

4. The Semen

Human semen is a complex cell suspension in a heterogeneous fluid produced by the male reproductive glands. Its primary role is to act as a buffering medium for sperm as it travels from the male reproductive canal to the female reproductive tract [10].

The semen components are delivered in sequential order. During ejaculation, the accessory sex glands release their fluids by contracting in an organ-specific order. Initial mucinous secretion – pre-ejaculate – lubricates the urethra, neutralises any residual traces of urine, and is secreted by the Cowper and periurethral glands. Simultaneous contraction from the epididymis and prostate comprises the following semen fraction: here, the maximum sperm concentration is found. Lastly, the seminal vesicles contribute the largest portion of the ejaculate [10]. After ejaculation, any remaining semen component in the male tract undergoes resorption or expulsion by urination [29].

4.1 Composition

Typically, the human ejaculate is about 3 ml in volume and exhibits an alkaline pH. Normal semen is a greyish-opalescent fluid (although many spermatozoa or leukocytes may result in a whitish appearance) with a density ranging from 1.043 to 1.102 g/ml [30].

Semen comprises cellular and acellular components (**Figure A.3**). The latter represents more than 99% of the total semen volume and is termed seminal fluid [10]. Cellular components are typically less than 1% of the ejaculate volume [29]. Sperm cells, epithelial cells of the urogenital tract, spermatogenic cells, and leukocytes constitute this fraction. The acellular fraction is an agglomeration of secretions produced mainly by the reproductive organs [10]. Interestingly, the seminal fluid components are not essential for fertilisation; however, they are necessary for sperm transport/maturation and enhance *in vivo* sperm fertilisation capacity [10]. The human seminal fluid contains diverse molecules ranging from organic to inorganic ions. The average protein concentration is 25 to 55 g/l, with albumin representing one-third of the total protein content [31, 32]. This albumin is of prostatic origin, while most proteins come from the seminal vesicles [10].

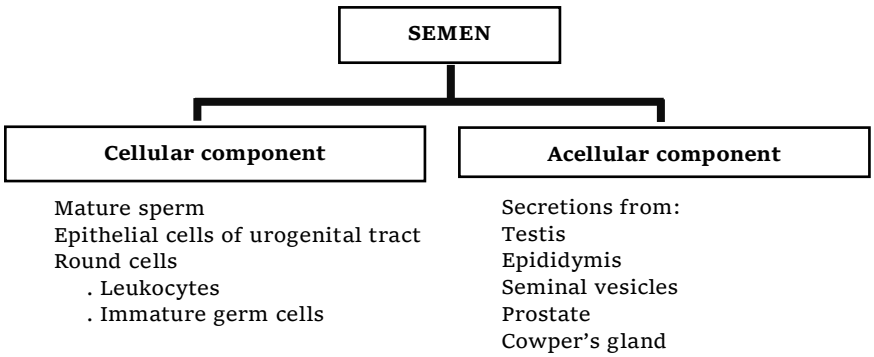


Figure A.3. Composition of human semen.

5. Male Infertility

According to Agarwal et al. [33], the worldwide prevalence of men experiencing infertility falls between 2.5% and 12%. Furthermore, Sharlip et al. [34] estimated that male factors alone account for 30% of infertility cases and are present in 20% of cases where male and female factors coexist. Male infertility rates vary between regions and populations [35]. These variations reflect the impact of lifestyle, socioeconomic, and environmental factors on human reproduction.

Throughout the years, a decline in male fertility has been reported by countless studies [36, 37], but most notably by Levine et al. [38]. They reported, from data collected over almost 40 years, from different geographic regions, that global sperm concentration suffered a significant downturn. Globally, 52.4% decrease in sperm concentration (a 0.7 million/ml/year drop in global sperm concentration) between 1973 and 2011, and 59.3% in total sperm count. The observed decline in sperm quality is undoubtedly partially associated with cryptorchidism, hypospadias, and testicular cancer [39]. However, environmental and lifestyle factors have also been shown to impact male reproductive health profoundly:

exposure to endocrine-disrupting chemicals [40, 41], pesticides [42], heat [43], diet [44], stress [45], smoking [46], exercise and body max index (BMI)[47]. Furthermore, advanced paternal age has also become a significant risk factor associated with male factor infertility [48–54].

The interdependencies of male and female reproductive functions demand a complete anamnesis, a careful physical examination followed by technical and laboratory investigations of the couple. In some cases (30-40%), when no male-associated factor can explain the impairment in sperm parameters, male infertility is referred to as idiopathic. No history of diseases affecting their reproductive health is present, and they show normal findings on physical examination and endocrine, genetic, and biochemical testing [55]. On the other hand, unexplained male infertility is the infertility of unknown origin associated with normal sperm parameters and partner evaluation. Unexplained infertility occurs in 20 to 30% of couples [56].

Male infertility can have underlying causes that span multiple levels within the body. However, the prominent disturbances are located in the testes, seminal ducts, accessory sex glands, and central structures such as the hypothalamus, pituitary, or other androgen target organs [3]. All or several structures mentioned above may be affected at any time by general and systemic diseases, thus affecting the male reproductive system. Although some fertility disturbances can be treated, for others, preventive therapies (for anomalies, such as testicular descent and infections) or symptomatic therapy (assisted reproductive techniques, ART) are the only possible course of action. As it does not eliminate the underlying cause of infertility, the latter is applied independently of the diagnosis and only based on semen parameters or sperm extractability [3].

II. OBJECTIVES

The purpose of this section is to provide a brief and straightforward overview of the current state of knowledge in the field of reproductive medicine and andrology, to provide a discussion on the wide range of factors responsible for male infertility, and to provide epidemiological data on male infertility in the Trás-os-Montes and Alto Douro region.

In summary, the following goals were defined: (1) review current knowledge on (male) reproduction and infertility and provide an epidemiological overview of male infertility, (2) provide a descriptive analysis of age, occupation, and lifestyle of males that attended fertility consultations at CHTMAD and critically assess and estimate the importance of the aforementioned factors on male infertility, (3) review critical aspects of sperm cells and semen characteristics that help in the estimation of male fertility potential, (4) provide a detailed protocol of routine laboratory tests used for male infertility diagnosis, discuss their importance and limitations, (5) offer a descriptive analysis of the main findings of the semen analysis throughout the years at CHTMAD, discuss apparent trends in sperm parameters and their significance, and, when possible, provide an explanation considering the male patient's characterisation, (6) provide the main cytogenetic and molecular findings during the internship period and discuss their relevance, and (7) present clinical cases of interest and discuss aspects regarding their aetiology, and consequences to the affected couple.

III. MATERIAL & METHODS

1. The Internship

The CHTMAD is a public business entity (EPE) created in February 2007, with its headquarters in the district of Vila Real. It arose from the merger of three public hospitals: Centro Hospitalar de Vila Real/Peso da Régua, EPE, the Hospital Distrital de Chaves and the Hospital Distrital de Lamego. Currently, four hospital units comprise the CHTMAD: Hospital de S. Pedro (Vila Real), Hospital Distrital de Chaves (Chaves), Hospital de Proximidade de Lamego (Lamego), and the Palliative Care Unit of Vila Pouca de Aguiar (Appendix A. The Trás-Os-Montes and Alto Douro Hospital Center (CHTMAD))[57]. The CHTMAD provides health care to the population in its area of influence, which includes the entire district of Vila Real, eight municipalities from the district of Viseu and the district of Bragança (in instances of absence or inadequacy of some clinical areas in their local health unit). As such, the CHTMAD serves a population of 375,000 citizens [57].

The andrology laboratory is currently located within the Genetics Laboratory, under the Department of Woman and Child of the Vila Real Hospital Unit. Here, routine basic semen analysis and any relevant cytogenetic and molecular investigations concerning infertility are performed.

The internship spanned 14 months, from September 2021 to November 2022, and encompassed various activities. These included conducting both conventional and advanced semen analysis, performing classical and molecular cytogenetic evaluations, investigating Y chromosome microdeletions (YCMD) (Section A), as well as studying the effects of cryopreservation on sperm parameters (Section B).

2. Study Participants & Design

From September 2021 to November 2022, men who underwent fertility testing at CHTMAD (study population A), as well as 13 semen donors (study population B), completed an anonymous short, discretionary, lifestyle self-assessment questionnaire (Appendix B. The Lifestyle Self-Assessment Questionnaire) on the day of semen collection. Participation in the study was voluntary and dependent on consent (Appendix C. Informed Consent). All samples were further anonymised (***Figure A.4***). In the case of study population A, 56 semen samples were analysed using conventional semen analysis. One sample underwent sperm aneuploidy testing (SAT), and nine samples were subjected to the Alkaline Comet Assay to assess DNA damage. Additionally, cytogenetic studies were conducted on eight infertile males, and YCMD testing was performed on five males. As for the study population of semen donors (population B), 13 semen samples were collected for semen analysis and DNA damage studies. Among these, two samples were further utilised for SAT (***Figure A.4***).

Simultaneously, a retrospective cross-sectional study was conducted in the CHTMAD andrology lab. Medical/laboratory records and data were reviewed. These records included information on lifestyle habits, semen analysis, cytogenetic studies, and molecular investigation results, covering 2010 to 2021 (study population C) (***Figure A.4***). In summary, the analysis encompassed 888 spermiograms along with their corresponding lifestyle self-assessment questionnaires, 491 cytogenetic investigations, and 27 YCMD studies reports (***Figure A.4***).

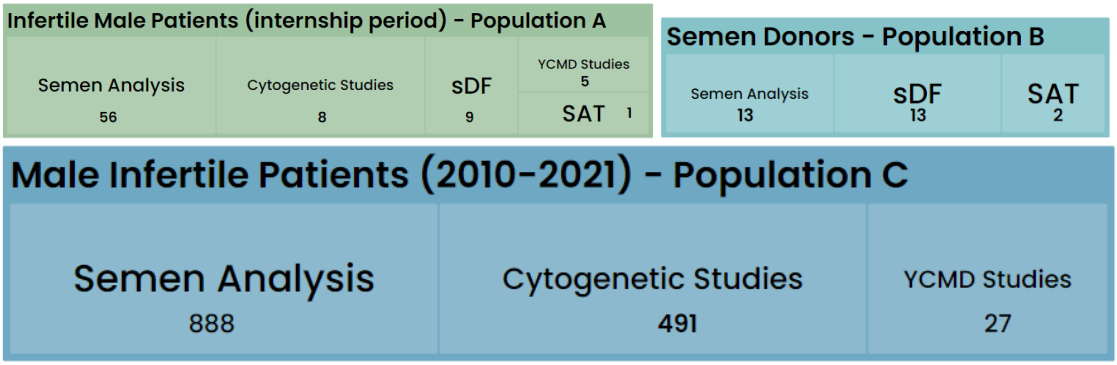


Figure A.4. Study populations, frequency and type of infertility tests conducted at the andrology laboratory. *Three populations were under analysis: population A, comprised of infertile male patients evaluated during the internship period; Population B, comprised of semen donors and lastly, population C comprised of infertile males assessed from 2010 to 2021. The type of analysis performed is described, as well as the corresponding number of samples for each assessment. sDF, sperm DNA fragmentation; SAT, sperm aneuploidy test; YCMD, Y-chromosome microdeletion.*

3. Semen Analysis Protocol

Deficiencies in sperm characteristics are associated with sperm underperformance. Therefore, in men with infertility, diagnosis requires an evaluation of the basic semen parameters: count, morphology, motility, and viability. Treatment decisions often depend on the results of semen analysis, and in clinical practice, it is viewed as the surrogate measure of male fertility [58–60]. However, semen analysis alone cannot distinguish fertile from infertile men [61].

Ejaculate analysis has been standardised by WHO and disseminated through the publication of the WHO Laboratory Manual for the Examination and Processing of Human Semen [30, 62]. In addition, WHO provides a threshold (lower 5th percentile from the distribution of values shown by semen samples of more than 1,800 men whose partner have conceived within 12 months) (**Table A.1**) and respective nomenclature to differentiate between normal and abnormal semen parameters (**Table A.2**) [30]. However, routine semen analysis yields normal semen parameters in 20% of couples [10]. In these cases, additional tests are necessary to determine specific disorders, otherwise undetected or challenging to assess by conventional semen analysis. Such tests are currently being implemented to help detect specific sperm dysfunction, predict fertilisation and pregnancy rates, and aid in selecting appropriate treatment [10, 30, 62]. However, usefulness is still highly debated for some of these tests, and standardisation is severely lacking [55, 62].

For the results of a semen analysis to prove valid and valuable, all aspects of ejaculate collection and examination must be performed under standardised procedures, as described by the WHO [30]. Ejaculate examination procedures are time-sensitive. Therefore, to ensure the quality of the examination, some assessments must be temporally divided to prevent the semen quality from being compromised by dehydration or fluctuations in temperature. Between 30 minutes and 1 hour after specimen collection, the following analysis must be performed: liquefaction and macroscopic appearance of semen, evaluation of sperm motility, sperm vitality assessment, pH measurement, evaluation of sperm concentration and smears to assess sperm morphology.

Table A.1. *Cutoff reference (5th percentile) and 50th percentile values for semen parameters from men whose partners achieved clinical pregnancy within 12 months of unprotected sexual intercourse. Adapted from WHO [30].*

Semen Parameters	**Percentile**	
	5th	50th
Volume (ml)	1.5	3.7
Vitality (%)	58	79
Progressive Motility (%)	32	55
Total Motility (%)	40	61
Concentration (10^6/ml)	15	73
Total Sperm Count (10^6)	39	255
Morphology (% normal forms)	4	15

Table A.2. *Nomenclature related to semen analysis. Adapted from WHO [30]*

Normozoospermia (N)	The total number (or concentration) of spermatozoa and percentages of progressively motile and morphologically normal spermatozoa are equal to or above the lower reference limits.
Azoospermia (AZ)	No spermatozoa in the ejaculate and in the centrifuged pellet.
Cryptozoospermia (C)	Spermatozoa were absent from fresh preparations but observed in a centrifuged pellet.
Oligozoospermia (O)	Total number (or concentration) of spermatozoa below the lower reference limit.
Asthenozoospermia (A)	Percentage of progressively motile spermatozoa below the lower reference limit.
Necrozoospermia	Low percentage of live (and high percentage of immotile) spermatozoa in the ejaculate.
Teratozoospermia (T)	Percentage of morphologically normal spermatozoa below the lower reference limit.
Asthenoteratozoospermia (AT)	Progressively motile and morphologically normal spermatozoa below the lower reference limits.
Oligoasthenozoospermia (OA)	Total number (or concentration) of spermatozoa and progressively motile spermatozoa below the lower reference limits.
Oligoasthenoteratozoospermia (OAT)	Total number (or concentration) of spermatozoa, progressively motile and morphologically normal spermatozoa below the lower reference limits.
Oligoteratozoospermia (OT)	Total number (or concentration) of spermatozoa and morphologically normal spermatozoa below the lower reference limits.

Subsequently, after a minimum of 4 hours, the smears are stained, and sperm morphology is assessed. For a detailed protocol, please consult Appendix D. Semen Analysis Protocol.

3.1. Macroscopic Evaluation

a) Liquefaction: sample liquefaction usually occurs in 15-30 minutes at room temperature (RT). The specimen container is therefore placed in an incubator (37°C) to facilitate liquefaction. After 30 minutes, if the specimen has a homogeneous appearance, the liquefaction is complete, and the semen analysis can proceed. If not, the container can remain in the incubator for another 30 minutes. When the ejaculates do not liquefy, mechanical mixing or enzyme digestion may prove necessary.

b) Appearance: the liquefied ejaculate has a cream/grey opalescent appearance. It may appear more translucid if the ejaculate has a low sperm concentration, yellowish in cases of a long abstinence period, and red-brown if red blood cells are present (haemospermia).

c) Volume**:** the volume of the ejaculate was measured directly by aspiration into a pipette.

d) Viscosity: the sample viscosity was assessed by gentle aspiration into a wide-bore plastic disposable pipette, followed by semen drop by gravity. Normal samples leave the pipette in tiny discrete drops. However, if a thread more than 2 cm long is visible, the viscosity of the ejaculate is classified as abnormal.

e) Odour: The odour of semen is typically unique and distinctive (*sui generis*). Therefore, variations in semen smell (especially urine or putrefaction) are of clinical importance.

f) pH: acidic prostatic secretion and alkaline seminal vesicular secretion determine the pH of semen. The only clinical interest in ejaculate pH is a low value (below 7,2).

3.2 Microscopic Evaluation

The sample must be well mixed before removing an aliquot for any assessment. Otherwise, separate aliquots can show marked differences in sperm motility, vitality, concentration, and morphology. A wet preparation is scanned at x100 magnification to provide an overview of the sample. The central goal is to determine whether there is an uneven distribution of sperm, sperm aggregation or agglutination, and the presence of mucus strands. The preparation is then assessed at x200 or x400 total magnification to determine the dilution for sperm concentration, the presence of round or epithelial cells, and the assessment of sperm motility.

a) Motility**:** according to WHO [30], sperm motility can be graded as progressive (PR) (sperm that move actively, linearly, or in large circles), nonprogressive (NP) (sperm that exhibit movement with an absence of progression), and immotile (IM) (sperm that show no movement). Two replicates are made, and 200 sperm are counted per replicate. The replica values are then checked to determine the difference's acceptability.

b) Concentration: the concentration of sperm ejaculate is determined under a microscope using *Neubauer's hemocytometer*. Depending on the number of spermatozoa counted, the sperm count is assessed in distinct chamber areas. Both chambers are used to assess sperm numbers. Only whole sperm (with heads and tails) are counted, and at least 200 in each chamber in the central grid must be counted. The eight peripheral grids are only used if fewer than 200 sperm are counted in the central grid. The difference between the replicate counts is determined, and if acceptable, the sperm concentration is determined. The total sperm count is calculated from the concentration of sperm and the volume of the ejaculate.

c) Sperm vitality**:** the vitality test allows immotile dead sperm to be distinguished from immotile live sperm. It is estimated by evaluating the membrane integrity of the cells through two methods (**Figure A.5**) : dye exclusion (damaged plasma membranes, a feature of dead cells, allow the entry of membrane-impermeant stains) and by hypoosmotic swelling (only cells with intact membranes, live cells, swell under hypotonic conditions). Two replicates and 200 sperm in each replicate must be evaluated. Replica values are again checked to determine the difference's acceptability.

. Dye exclusion test [63] (**Figure A.5**): live spermatozoa exhibit white heads, while dead spermatozoa have red-stained heads.

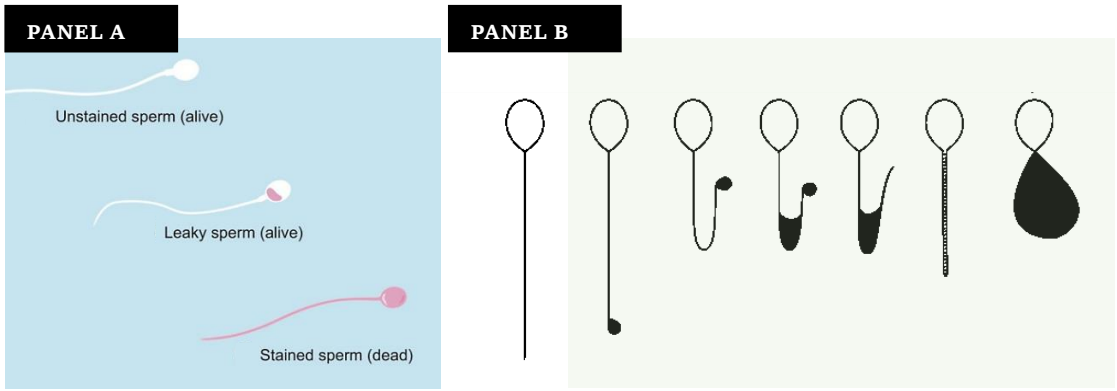


Figure A.5. Sperm vitality test*. Panel A – Schematic representation of alive and dead sperm: white or faint pink spermatozoa indicate alive spermatozoa; red or dark pink heads indicate non-vital sperm cells. In instances where the stain is limited to part of the neck region only, this is considered a leaky membrane and the sperm cell is scored as alive. Adapted from Agarwal et al* [63]*. Schematic representation of human spermatozoa under hypoosmotic stress. Swelling in tails is indicated by black area. Alive spermatozoa are shaded green. Adapted from WHO* [30].

Hypoosmotic swelling (HOS) test: live spermatozoa swell within 5 minutes in hypotonic medium, but only after 30 minutes do all flagellar shapes stabilise. Live sperm are recognised by swelling of the tail. This, however, can assume many forms, as represented in **Figure A.5**.

d) Morphology**:** infertility assessment is heavily based on sperm morphology, despite the difficulty of its assessment (due to lack of objectivity, variation in interpretation, and poor performance in external quality control assessments). A wide range of distinct morphological sperm is a characteristic of all human ejaculates, so the term 'normal' sperm refers to the morphology most sperm exhibit in postcoital endocervical mucus [64, 65] and on the surface of the zona pellucida [66, 67]. For the morphology evaluation, 200 spermatozoa are assessed. Not only is the determination of the proportion of 'normal' spermatozoa vital, but it is also crucial to evaluate the specific morphology of the head, neck/midpiece, and tail and the presence of abnormal cytoplasmic residues. The WHO defines the criteria for classifying sperm morphology. A normal sperm must simultaneously have a typical head, midpiece, and tail. All other combinations are considered abnormal. The teratozoospermia index (TZI) (the sum of all abnormalities divided by the sum of abnormal spermatozoa) is calculated. It ranges from 1.0 to 4.0. This reflects the minimum and maximum defects found per abnormal sperm: one each for the head, midpiece, principal piece, and excess residual cytoplasm.

4. The Comet Assay

Various processes, from defective spermatogenesis and oxidative stress to genital tract inflammation, can trigger sperm DNA fragmentation (sDF) [68]. Multiple studies indicate that embryo development, implantation, and pregnancies (through natural and assisted reproduction) are affected by sDF but not the fertilisation capacity [69–72]. Extensive sDF has been proposed to be associated with normozoospermic but infertile individuals [62, 73]. Furthermore, sperm DNA damage is also correlated with semen parameters such as reduced progressive motility and abnormal head shape [74] and has gradually become an important prognostic and diagnostic marker [75]. Several assays are currently available to clinicians to assess DNA damage. However, not only are these approaches not standardised, but there is still a debate over which test has the highest diagnostic value.

For sperm DNA damage measurement (single and double-strand breaks), single-cell electrophoresis – the Comet assay – was used.

The Comet assay allows the evaluation of sDF in individual sperm. Differential migration of broken DNA strands, when subjected to an electric field, is the fundamental principle behind this method: smaller fragmented strands migrate faster than the nonfragmented, intact strands. Briefly, sperm are embedded in an agarose matrix; lysis is promoted under high salt conditions, and with the aid of a detergent (due to the nature of chromatin condensation in sperm, the addition of reducing agents such as DTT and a proteinase is also needed). Nuclear proteins are thus removed, generating a nucleoid structure where, under alkaline conditions, the double-stranded DNA can migrate towards the anode. In the following electrophoresis step, the broken DNA strands migrate, generating the "comet tail"-like pattern: intact DNA is found in the comet's head, and the broken strands make up the tail. The assay has many variations, depending on the type of damage of interest to be assessed [76]. Here, the following variation was employed:

. Alkaline Comet Assay (ACA): sperm chromatin is uncoiled and denatured in an alkaline buffer, and alkali-labile sites are converted into DNA breaks. This variation allows for the scoring of sDF composed of single and double-strand breaks. However, it does not allow for the differentiation between single and double-strand breaks.

Following electrophoresis, neutralisation and staining, DNA damage was evaluated by classifying comets into five categories (0-4) according to their appearance [76]. The number of cells scored per gel was 100. A detailed protocol can be found in Appendix E. Alkaline Comet Assay (ACA) Protocol**.**

Lastly, Azqueta et al. [77] argue that there is a reasonable level of agreement between the visual score and automated analysis. Thus, all scores obtained for ACA were converted to a percentage of DNA in the tail. This conversion was necessary to ensure accurate interpretation and facilitate comparison with the existing literature.

5. Sperm Aneuploidy Test (SAT)

Following the completion of meiosis, sperm have a haploid set of chromosomes. However, even in men with a normal karyotype, disturbances in chromosome segregation during meiosis can result in the gain (disomy) or loss (nulisomy) of a chromosome. Once again, even among fertile couples, aneuploidies in sperm are to be expected, although at very low rates. Extrapolations from tested chromosomes point to a 3-5% aneuploidy rate among males with proven fertility or normozoospermic semen samples [78, 79]. However, abnormally high levels of aneuploid sperm are commonly observed in men with spermatogenic failure, oligozoospermia or oligoasthenozoospermia and in men whose partner has experienced recurrent pregnancy loss (RPL) or with a previously failed ART [52]. Furthermore, increased sperm aneuploidy is associated with increased levels of sDF [80]. Carriers of structural (balanced) or numerical alterations constitute the most significant group of individuals at a higher risk of sperm aneuploidies (owing to the accumulation of unbalanced segregation products in the sperm nucleus)[81].

Analysis of the complete haploid complement of chromosomes in sperm, although the most informative approach, is currently cost-prohibitive. Therefore, aneuploidies for chromosomes X, Y, 13, 18 and 21 are the most tested. Mainly because aneuploidies involving these chromosomes are associated with viable but affected offspring. Sperm aneuploidy in fertile men is rare; however, a clear threshold for fertile and infertile men has not been established

[82]. Nonetheless, this methodology proves helpful in the genetic counselling of affected couples and aids couples in making informed reproductive decisions [52, 82].

Fluorescent in situ hybridisation (FISH) is the cytogenetic assay used to assess the frequency of sperm chromosomal abnormalities. It is based on the ability of marked, single-stranded DNA sequences (probes) to selectively bind to the complementary single-stranded chromosomal regions of interest. For this purpose, chromosomal DNA is denatured and hybridised with the probes (marked with a fluorochrome). In regions where hybridisation has been successfully performed, fluorescence is detected with a microscope equipped with the appropriate filters. The FISH assay has a higher resolution than conventional cytogenetics (up to 100Kb) and can be applied to interphase nuclei (as is the case for sperm aneuploidy tests)[83]. In particular, the chromosome-specific probes are identified by the colour of their attached fluorochrome, and the nuclei are scored for the presence of signals for each of the probes used.

First, raw semen samples were washed and fixed on microscope slides to achieve the ideal sperm concentration for scoring. Nuclear decondensation and dehydration of the sperm followed. The probe mixture was then added to the slide; a coverslip was dropped and sealed to the slide with rubber cement. The FISH protocol was carried out using probes for chromosomes 13, 18, 21, X and Y while strictly adhering to the time and temperatures specified by the probe manufacturers for denaturation, hybridisation, and post-hybridisation washes.

Slides were manually scored following a strict criterion to eliminate subjective variation, according to WHO guidelines [62]: (1) observed signals should be a single spot with little or no noise;(2) overlapped spermatozoa or sperm heads where a well-defined boundary is not apparent are not counted; (3) in cases of disomy or diploidy, all signals must be equal in size and at least 1.5 domains separated from each other; (4) signals must be within the nucleus. Although not stated in the WHO laboratory manual, there is a relative consensus that a minimum of 1,000 to 5,000 sperm cells per sample for each chromosome must be scored [78, 84, 85]. However, here only 200 sperm were manually scored for each chromosome. A comprehensive and detailed protocol for the conducted sperm aneuploidy test is present in Appendix F. Sperm Aneuploidy Test (SAT).

6. Conventional Cytogenetics

In infertility studies, chromosomal disorders are often numeric alterations (sex chromosomes) and structural aberrations. The frequency of chromosomal abnormalities increases in close association with the severity of testicular deficiency. Men with less than 5 million sperm cells/ml show a 10-fold higher incidence (4%) of autosomal structural abnormalities than the general population [86, 87]. However, the group with the highest risk is men with non-obstructive azoospermia. Normozoospermic infertile men also have an increased risk of a cytogenetic abnormality [88]. As such, karyotype analysis is indicated in men with azoospermia or oligozoospermia (<10 million sperm cells/ml), and, regardless of sperm concentration, karyotype analysis is also requested if there is a family history of recurrent spontaneous abortions, malformations, or cognitive impairment [55].

The study of chromosomes through conventional cytogenetic techniques involves analysing the banded pattern of metaphase chromosomes under a light microscope. Karyotyping is a laboratory procedure used to examine an individual's chromosomes. It involves pairing and ordering all the chromosomes of an organism, providing a genome-wide snapshot of an individual's chromosomes. Infertility studies routinely use this technique to detect aneuploidies and structural abnormalities with a resolution of 5-10Mb [83].

The methodology for a conventional cytogenetic investigation (for infertility studies) involves several steps. First, peripheral blood lymphocytes are collected from the individual. The cells are then grown in culture to increase their number (culture initiation and maintenance). Cells are then treated with a chemical to stop cell division at metaphase, where the chromosomes are the most visible and condensed (cell harvesting). Lastly, chromosomes are stained (chromosome staining) and visualised under a microscope to determine their number, size, shape, and banding pattern (chromosome imaging and analysis).

6.1 Specimen Collection, Culture Initiation & Maintenance

Peripheral blood samples are collected in sterile vacuum tubes containing lithium heparin. If not processed immediately, the samples are refrigerated and processed within 24-48 hours after collection. Specimens are grown and maintained in an aqueous culture medium – balanced salt solutions with various additives such as salts, glucose, amino acids, vitamins, growth factors, and a buffering system to maintain proper pH [83]. Furthermore, many media employ phenol red as a pH indicator. A too-acidic medium turns yellow, while a too-basic one turns pink or purple. Commercial media are often sold incomplete, meaning that they must be supplemented with crucial additives such as:

. L-Glutamine – an amino acid essential for cell growth;

. Foetal bovine serum (FBS) – a culture medium supplementation of 10-30% FBS allows maximum cell growth;

. Antibiotics – penicillin/streptomycin and kanamycin are added to the culture medium to hinder the growth of microorganisms.

Lymphocytes are free-floating cells displaying no anchorage dependency for growth, and, as such, suspension culture in sterile centrifuge tubes is often the culture method of choice. Furthermore, blood samples contain (in normal circumstances) only mature lymphocytes that do not spontaneously undergo cell division. Therefore, they require the addition of mitogens to the cell medium. Phytohemagglutinin, an extract of red kidney beans, primarily stimulates T-lymphocyte division [83]. Cell division usually starts in the next 48 hours, with more mitotic waves every 24 hours. For peripheral blood culture, a 72-hour culture period is considered optimal. To obtain high-resolution chromosomes, cultured blood lymphocytes are usually synchronised and harvested earlier in the cell cycle to prevent obtaining chromosomes at their most contracted state. Such methods involve the addition of methotrexate (MTX) to peripheral blood cultures before the harvest phase. MTX is a folate antagonist that, through competitive inhibition, hinders the activity of folate-dependent enzymes and the synthesis of purine and pyrimidine required to produce DNA and RNA [89]. Cell division is effectively blocked in the G1/S stage and is only rescued by adding thymidine. Five hours later, the harvest is performed: the accumulated cells are in prometaphase, and the chromosomes are longer and less contracted [83].

6.2 Cell Harvesting

The term 'harvest' refers to the procedure of collecting cells during metaphase, subsequently subjecting them to a hypotonic treatment and fixation, followed by chromosome spreading on glass slides [83].

First, a mitotic inhibitor is used to arrest cells in metaphase. Colcemid binds to tubulin proteins and hinders the formation of the mitotic spindle or destroys those already present. In this manner, sister chromatids cannot be separated, anaphase is effectively blocked, and cells remain locked in metaphase. The exposure time to colcemid is critical, as the chromosomes condense as they progress through metaphase. However, a longer exposure time means more cells can be collected in metaphase. To avoid the necessary trade-off between quantity and quality, chemical elongation is often performed. In particular, the high-

yield harvest of prometaphase chromosomes is made possible by adding intercalating agents, such as ethidium bromide, preventing or delaying chromosomal condensation [83].

Following exposure to colcemid, a hypotonic solution is added to the cells. The lower salt concentration (compared to the cell's cytoplasm) allows water to enter the cell by osmosis and causes cell swelling (necessary for proper chromosome spread). Next, a fixative solution is used to stop the hypotonic solution treatment, preserve the cell's swollen state, remove lipids, and denature proteins. This allows for a fragile cell membrane, further facilitating chromosome spreading [90].

Chromosome spreading is the final event of the harvesting phase. A well-spread chromosome slide has metaphases with minimal overlap of the chromosomes and no visible cytoplasm. After fixation, the swollen, fragile cells are dropped onto the glass slides, and the fixative spreads and evaporates. Downward pressure is exerted on the cell by the fixative's surface tension. As the fixative evaporates, the cell membranes are stretched further, and the cells become flatter. Cells and chromosomes become more spread the longer the evaporation takes. Ambient temperature, humidity, the length from which the cells are dropped, the use of steam, and other variables affect the spreading of chromosomes [91]. Thus, test slides are always made and checked for metaphase quality. After chromosome spreading, the slides are aged overnight at 60°C to enhance chromosome banding. Slide ageing allows the (partial) removal of water from the chromatin necessary for a good banding. Excess water prevents the chromatin from acquiring the desired dark and light staining regions [91].

6.3 Chromosome Staining & Banding

Under a light microscope, chromosomes are colourless; thus, staining/banding methods have been developed to aid in visualisation. Banding techniques are divided into two groups: those that produce alternating bands along the length of the entire chromosome and those that specifically stain a band or region of some or all chromosomes. These methods allow for the unambiguous identification of each human chromosome by producing unique patterns for each homologous set, and, in turn, chromosome abnormalities can be ascertained with relative ease and confidence (within the typical resolution boundaries of classical cytogenetics) in clinical and research settings. Although in use for almost five decades, the mechanism of chromosome banding and staining is still not fully understood. However, they remain vital for cytogenetic studies [83, 91]. A three-letter code is used to describe the various banding techniques. The first denotes the type of banding (G, Q, R, C); the second describes the general technique (H, heating; T, trypsin; B, barium hydroxide); and the last letter indicates the stain used (G, Giemsa; L, Leishman; Q, quinacrine). These different staining techniques reflect DNA replication's packaging, base composition, and timing [83].

6.3.1 G-Banding (GTL)

Sumner et al. [92] introduced the G-bands, the most used banding technique for chromosome analysis. Although the histochemical basis behind this stain remains elusive, the dark G bands represent late-replicating, AT-rich, heterochromatic regions of the chromosome, while the light G bands are early-replicating, GC-rich, euchromatic regions [93, 94]. Trypsin treatment is applied to the slides so the dye can differentially bind to the chromatin. Next, the enzyme is washed off, the stain (in this case, Leishman) is applied, rinsed off, and the slide is dried. The banding quality strongly depends on the exposure time to trypsin. Therefore, one slide is always banded first as a trial slide and is judged on trypsin time and concentration. Fuzzy chromosomes with indistinct bands and little contrast are usually under-trypsinised, whereas over-trypsinized chromosomes appear to have frazzled ends with sharp bands and too much contrast between them. On the other hand, adequately stained chromosomes exhibit an appropriate amount of contrast between bands echoed by a range of grey values throughout the length of the chromosome.

6.3.2. C-Banding (CBL)

Unlike G-banding, C-bands are not used for chromosome identification. Instead, they are only used to visualise constitutive heterochromatin in the centromeres and the secondary constrictions of the chromosomes. Constitutive heterochromatin (20% of the human genome) contains few, if any, structural genes, is seldom transcribed, is highly polymorphic, replicates late and is comprised of highly repeated satellite and non-satellite DNA sequences [91]. Despite being located around the primary constriction of all human chromosomes, it is most abundant at the secondary constrictions of chromosomes 1, 9, 16 and the long arm of the Y chromosome. The C-band technique is used primarily to aid in identifying polymorphic variants (since they have no known phenotypic impact [95]) on chromosomes. The acidic, basic treatment extracts the DNA purine bases in CBL (C-bands by barium hydroxide, using Leishman). It selectively denatures euchromatin fragments washed away by incubation in a warm salt solution. Because constitutive heterochromatin resists this degradation, the Leishman stain can only bind to these remaining chromatin fragments. Therefore, pale chromosomes, selectively stained in their constitutive heterochromatin regions, are the result [83].

6.4 Chromosome Imaging & Analysis

Chromosomes, after staining, are visualised as consisting of a series of light and dark segments. Therefore, in the 1971 Paris Conference [96], a chromosome band was defined as a chromosome segment distinct from adjacent segments appearing darker or lighter by one or more staining techniques. Since this banding pattern is specific for each pair of chromosomes, the International Standing Committee on Human Cytogenetic Nomenclature established a standardised map (ideogram) and nomenclature [91]. This system accurately describes chromosome abnormalities and sets the universal standard for cytogenetic nomenclature [95].

Here, under high power, visual search and identification of metaphase chromosomes was accomplished by autonomous slide loading, scanning, and capture powered by Leica CytoVison computer imaging programme (Leica Biosystems 1nc., Vista, CA, USA), followed by manual selection: well spread, with good size and resolution, and metaphases with few to no overlaps were settled upon. Subsequently, the karyogram was prepared and enhanced using the built-in tools in the software. Lastly, a clinical report containing the patient's karyotype (according to the cytogenetic nomenclature – ISCN 2020 [97]), banding resolution, number of cells examined and microscope coordinates of the captured metaphase spread image were recorded in the patient's file and made available to the physician. For patients with a history of infertility and/or repeated miscarriages, ten metaphases were analysed. If low-grade mosaicism was suspected, more metaphases were examined.

7. YCMD Studies

The structure of the Y chromosome is of pivotal importance for male infertility studies: the genes necessary for spermatogenesis and testis development are located on the long arm of the Y chromosome [98]. During meiosis, only its pseudoautosomal regions recombine with the X chromosome. Its male-specific region contains 78 protein-coding genes and comprises 95% of the chromosome. YCMDs occurs in 11-18% of azoospermic men and 4 to 14% of cases of oligozoospermia [99]. Genetic studies on the Y chromosome focus on the azoospermia factor (AZF) region, located on the long arm of the Y chromosome (Yq11). The AZF region has 31-Y specific genes, and 14 are protein-coding. Three separate regions comprise this area (AZFa, AZFb and AZFc), and microdeletions of these areas (caused by intrachromosomal

recombination events between homologous repetitive sequences [100]) give rise to different phenotypes [101]: a) AZFa deletions cause azoospermia and give rise to Sertoli cell-only syndrome [102]; b) deletion of AZFb (and AZFbc) gives rise to azoospermia, but through an arrest of spermatogenesis at the primary spermatocyte stage [102]; c) AZFc microdeletions do not uniformly result in azoospermia, instead, a wide range of phenotypes can be seen: some normozoospermic men display partial deletions in this region, while complete deletions can result in an oligo or azoospermia [102]. Such deletions are thought to arise *de novo and* affect one in 5,000 males [103]. They represent one of the most frequent structural chromosome anomalies [103] and reflect Y chromosome instability [104].

Subsequently, for ART, the detection of these deletions is critical, as they are associated with different sperm retrieval rates (35% for azoospermic men with AZFc deletion and a low retrieval rate for men with AZFbc and AZFb deletions [105].

Molecular diagnosis of YCMD was accomplished through 4-colour (FAM/VIC/ROX/Cy5) real-time polymerase chain reaction (qPCR) analysis of sequence-tagged site (STS) markers within specific AZF regions. Due to Taq polymerase's 5'-3' exonuclease activity, this approach is based on fluorescence detection following the cleavage of a dual-labelled probe during hybridisation to the corresponding target sequence. Thus, the fluorescence detected in the quantitative PCR thermal cycler is directly proportional to the released fluorophore and the amount of DNA template present in the PCR reaction mixture [106] (Appendix G. YCMD Studies). This methodology comprises four major processes: (1) sampling, (2) DNA extraction, (3) specimen preparation, (4) PCR amplification, and (5) data analysis.

Briefly, 2.5 ml of venous blood was collected in a tube containing ethylenediamine tetraacetate (EDTA) and followed by automated nucleic acid extraction and purification (based on silica membrane) using the QIAamp® DNA Mini QIAcube Kit (Qiagen, Dusseldorf, Germany). qPCR was performed according to the manufacturer's instructions of the YCMD detection kit (Y6) (Tellgen Biotechnologies Inc., Shanghai, China) and using the LineGene 9600 Plus QPCR system (Bioer, Hangzhou, China). A total of six classic STS primers were used to analyse the AZFa, AZFb, and AZFc subregions [107] (Appendix G. YCMD Studies). In detail, for each sample, 50 ng of human genomic DNA was used as a template in two 25 μL reaction mixes (each containing three specific primers and probes). After an initial denaturation step of 5 min (95°C), PCR amplification was performed throughout 35 cycles at 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. The final elongation step was performed at 72°C for 5 min. For each PCR run, positive and negative control samples were prepared. Lastly, data analysis and interpretation were performed using LineGene 9600 Plus software. The absence of an S-shaped amplification curve and a Ct superior or equal to 32 indicates the deletion of the region in question. The complete deletion in a sample is observed by a lack of amplification for the two specific markers in that region (Appendix G. YCMD Studies).

8. Statistical Analysis

Data analysis was performed in R statistical software (version 4.2.2; R Foundation for Statistical Computing, Vienna, Austria). Descriptive statistics were used to visually represent the data and their characteristics. The geometric mean (unless when specified) and respective range for each variable are shown due to highly skewed data.

IV. RESULTS

1. Characterization of the infertile Male Patients at CHTMAD (population A)

1.1 Self-reported lifestyle behaviours

From September 2021 to October 2022, 56 infertile men (study population A) underwent semen analysis at the CHTMAD andrology laboratory. The patients were, on average, 36.7 years old, suffered from primary infertility (93%) and had an excess weight (BMI of 26.1 kg/m^2) (**Table A.3**, and **Figure A.6**). Concerning the age distribution of these patients, only 11% reported being under 30 years of age and 27% over 40 years of age. For self-reported BMI, obesity was present in 16% of the individuals; 47% were pre-obese, while 37% exhibited healthy weight. Furthermore, 45% exercise 1 to 2 times a week, 68% report no smoking habits, and 66% do not regularly drink alcohol. Only 30% report environmental or occupational exposure to relevant agents, such as pesticides, heat, radiation, and chemical paints (**Figure A.7**). The following eating habits were also detailed: 77% eat 3 to 4 meals a day, and most eat red meat (54%), vegetables (38%), and fruits (30%), 3 to 4 times a week. Cereals have the lowest representation among the food items consumed. Most patients (51%) report cereal consumption with a frequency equal to or less than twice a week.

Table A.3. *Main populational traits of infertile male patients at CHTMAD during the internship period.*

AGE (years)	BMI (kg/m²)	Meals (%)			Physical Exercise (%)		Smoking Habits (%)			Alcohol Consumption (%)		
		Times/day			Times/week		cigarettes/day			Drinks/day		
		≤2	3-4	≥5	1-2	>2	5-10	10-20	>20	1	2-4	>4
36.7	26.1	8	77	15	45	25	14.3	16.1	1.8	16	9	9

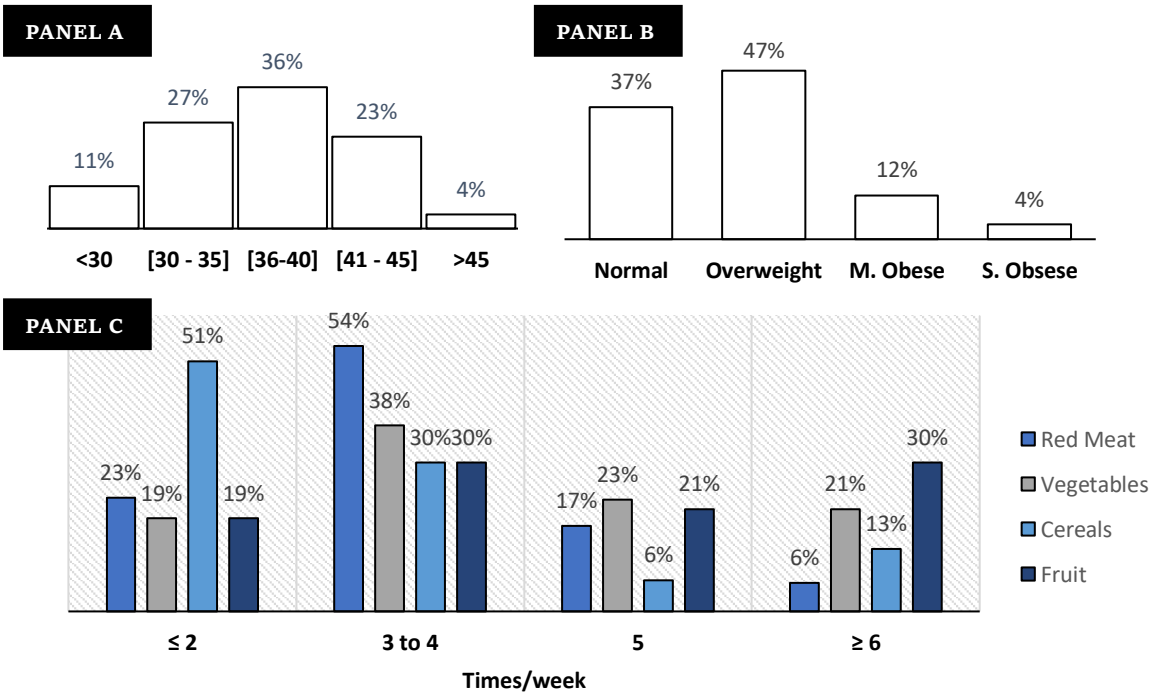


Figure A.6. Age and BMI distribution, and dietary habits for population A*. Panel A – age distribution. Panel B – BMI distribution. M. Obese – Moderate obesity; S. Obese – Severe obesity. Panel C – Dietary habits.*

Regarding occupation (**Figure A.7**), 31% of men are skilled service employees (bakers, drivers, bartenders, servers, etc.), 23% are skilled industrial employees (welders, automotive painters, carpenters, etc.), 21% are professionals (a group containing nurses, engineers, teachers, psychologists, etc.), 15% technicians, 8% are farmers, and 3% businessmen/entrepreneurs.

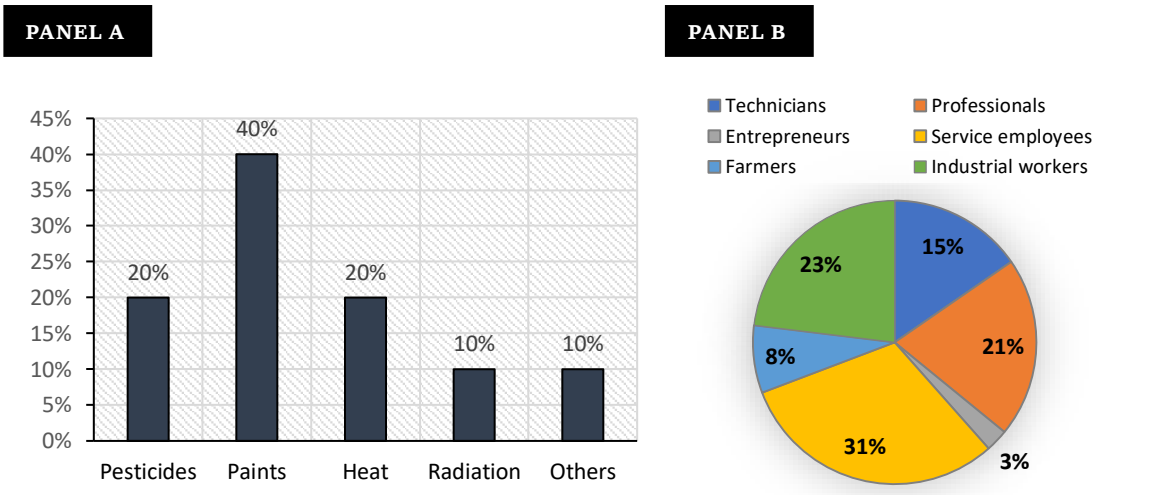


Figure A.7. Reported environmental exposure and frequency of described professions for the infertile males that underwent semen analysis during the internship. *Panel A – Environmental exposure to relevant agents. Panel B – Reported occupations.*

1.2 Semen Analysis

Thirty per cent of the 56 males subjected to analysis produced normozoospermic (N) semen samples (**Table A.4**). The most commonly detected abnormality was teratozoospermia (T) (28.6%), followed by oligozoospermia (O) (21.4%), oligoteratozoospermia, and azoospermia (AZ) (both present in 7.1% of cases). Asthenozoospermia (A), oligoasthenoteratozoospermia (OAT), and criptozoospermia (C) were found in only one sample (1.8%). These infertile males did not produce asthenoteratozoospermic (AT) and oligozoasthenozoospermic (OA) samples. When oligozoospermia was present (alone or in combination with other abnormalities) in almost half of the samples (47%), the displayed sperm concentration was 5 million/ml or less – severe oligozoospermia. And in this cohort of samples, only one sample (13%) had sperm concentration greater than 2 million sperm cells/ml. Lastly, mild necrozoospermia (from 40% to 58% vitality) was detected in only two samples.

Table A.4. *Semen profile for population A*

	Semen Sample Classification										
Frequency	A	AT	AZ	C	N	O	OA	OAT	OT	T	Total
N	1	0	4	1	17	12	0	1	4	16	56
%	1.8	0.0	7.1	1.8	30.4	21.4	0.0	1.8	7.1	28.6	100

A, asthenozoospermia; AT, asthenoteratozoospermia; AZ, azoospermia; C, criptozoospermia; N, normozoospermia; O, oligozoospermia; AO, oligoasthenozoospermia; OAT, oligoasthenoteratozoospermia; OT, oligoteratozoospermia; T, teratozoospermia.

Table A.5 provides a summary of the mean (and range) results for the main semen parameters in analysis. On average, the duration of abstinence was 2.8 days, and the males produced an average ejaculate volume of 2.8 ml, with a PR motility of 52% and 66% for total motility. On the other hand, the concentration averaged 30.6 million sperm cells/ml, and the total sperm count was 87.9 million sperm cells. It should be noted that these motility and concentration

values exceed the threshold set by WHO[30]. Regarding morphology, the frequency of sperm cells with normal morphology was found to be, on average, 3.5% per ejaculate. This is the first and only parameter below the WHO threshold. The semen parameters for each male patient of study population A can be found, in detail, in Appendix H. Semen Parameters of Population A.

Table A.5. *Semen parameters for study population A.*

		Population A			5th perc. WHO	Δ (%)
		Mean	Min	Max		
Abstinence period (days)		2.8	2.0	4.0		
Volume (mL)		2.8	1.0	7.4		
Vitality (%)		73.8	39.0	90.0		
Motility (%)	IM	28.6	12.0	72.0		
	NP	11.7	5.0	25.0		
	PR	52.1	8.0	81.0	32.0	+63
	Total	66.4	28.0	88.0	40.0	+66
Sperm Count (x10^6)	Conc. (10^6/ml)	30.6	30.6	150.0	15.0	+104
	TSC	87.9	6.6	685.2	39.0	+125
	TMSC	58.3	3.7	561.9	-	
	TPMSC	45.8	2.3	520.8	-	
Morphology	% NormaL	3.5	1.0	8.0	4.0	-12.5
	TZI	1.4	1.1	1.9		

Perc., percentile; Min, minimum; Max, maximum; Δ-Variation, in percentage, obtained from the following formula: (pop. A mean- 5th percentile)/5th percentile; IM, immotile; NP, Non-progressive; PR, progressive; TSC, total sperm count; TMSC, total motile sperm count; TPMSC, total progressive motile sperm count; TZI, teratozoospermia index.

1.3 Sperm DNA Fragmentation & Sperm Aneuploidy Test

Nine samples were subjected to DNA damage evaluation by ACA, and one sample to SAT. The sDF values are shown in **Table A.6**. The sample provided by patient nº4 was further used for SAT. The infertile men included in the study had an average age of 36.3 years and an sDF rate of 65.9%. Among the samples provided, 45% showed abnormalities, including one oligozoospermic sample, one oligoasthenozoospermic sample, and two teratozoospermic samples. These samples were obtained from men with an average age of 34.5 years and exhibited a 64.2% sDF rate. On the other hand, normozoospermic samples were obtained from older infertile men with an average age of 37.8 years and showed the highest sDF rate (67.4%).

Regarding the SAT, sample nº4 showed an aneuploidy rate of 4.5-7.5%. The distribution of aneuploidies for the different chromosomes analysed is shown in **Table A.7**. Disomy was the most common type of aneuploidy found. The disomy rate for chromosome 21 was eight times higher than that of chromosome 13 (4% compared to 0.5%). In the case of sex chromosomes, an XYY sperm cell was observed, while the disomy rate (all XY sperm cells) was six times higher than that of chromosome 18 (3% compared to 0.5%). Nulisomy was observed for chromosomes 18 and 21, each at a rate of 0.5%. Diploid sperm cells, containing two copies of chromosomes 13 and 21, were present at a rate of 2.5%. From the analysis of 200 sperm cells, specifically for chromosome 18 and sex chromosomes, a total of 191 cells showed a haploid set of chromosomes, accompanied by an X/Y ratio of 1.38.

***Table A.6**. ACA DNA damage evaluation for nine individuals in population A*

		sDF (%)	Age (years)
***N*= 9**	**Mean*±SD**	**65.9±20.7**	**36.3±5.8**
	Sample Classification		
1	N	53	38
2	N	78.4	30
3	N	80.5	35
4	N	69	40
5	N	56.1	46
	Mean*	**67.4**	**37.8**
6	O	74.6	26
7	OAT	81.6	38
8	T	81.5	39
9	T	18.9	35
	Mean*	**64.2**	**34.5**

*Arithmetic mean; SD, standard deviation; N, normozoospermia; O, oligozoospermia; OAT, oligoasthenozoospermia; T, teratozoospermia. The shaded grey sample underwent SAT.

***Table A.7**. Aneuploidy rates for sex chromosomes and chromosomes 13, 18 and 21.*

Chr. 13 (%)		**Chr.21 (%)**		**Diploidy (%)**	**Aneuploidy (%)**		
Nulisomy	Disomy	Nulisomy	Disomy				
0	0.5	0.5	4	2.5	7.5		
Chr. 18		**Sex chromosomes (%)**			**Aneuploidy (%)**	**Haploid (%)**	
Nulisomy	Disomy	Nulisomy	Disomy	Trisomy		X	Y
0.5	0.5	0	3	0.5	4.5	58	42

1.4 Cytogenetic and Molecular Studies

The eight infertile men subjected to cytogenetic studies exhibited a normal karyotype (46,XY). No deletions of the Y chromosome were detected in the five infertile patients studied.

2. Characterization of the semen donors (population B)

2.1 Self-reported lifestyle behaviours

Study population B consisted of 13 semen donors. On average, these donors were 25 years old and had a healthy weight with an average BMI of 24 kg/m^2 (**Table A. 8**). However, it should be noted that the age and BMI distribution in this group is highly skewed. Only 15% of the donors were over the age of 30, while 70% of the donors were men under or equal to the age of 25 years. In terms of BMI, 73% of the individuals fall into the category of healthy weight, 18% are overweight, and a half (9%) are classified as (moderate) obese (**Figure A.8**). Among these donors, 73% reported engaging in regular physical activity, with most (64%) indicating a frequency of more than twice per week. Additionally, an equal share of men (36%) reported daily smoking habits and daily alcohol consumption. Regarding the reported eating habits, 55% of the participants stated that they eat meals 3-4 times a day. Red meat and fruits were consumed the least – 45% report consumption up to twice a week. Vegetables and cereals, in contrast, were reported to be consumed more frequently (3 to 4 times a week by 55% and 45% of the participants, respectively). Lastly, no occupational or environmental exposure to relevant agents was reported among the participants. Furthermore, all men included in the study were students.

Table A. 8. *Main populational characteristics of semen donors (population B).*

Age (years)	BMI (kg/m²)	Meals (%)			Physical Exercise (%)		Smoking Habits (%)			Alcohol Consumption (%)		
		Times/day			Times/week		cigarettes/day			Drinks/day		
		≤2	3-4	≥5	1-2	>2	5-10	10-20	>20	1	2-4	>4
25.0	24.5	36	55	9	9	64	18	9	9	27	9	0

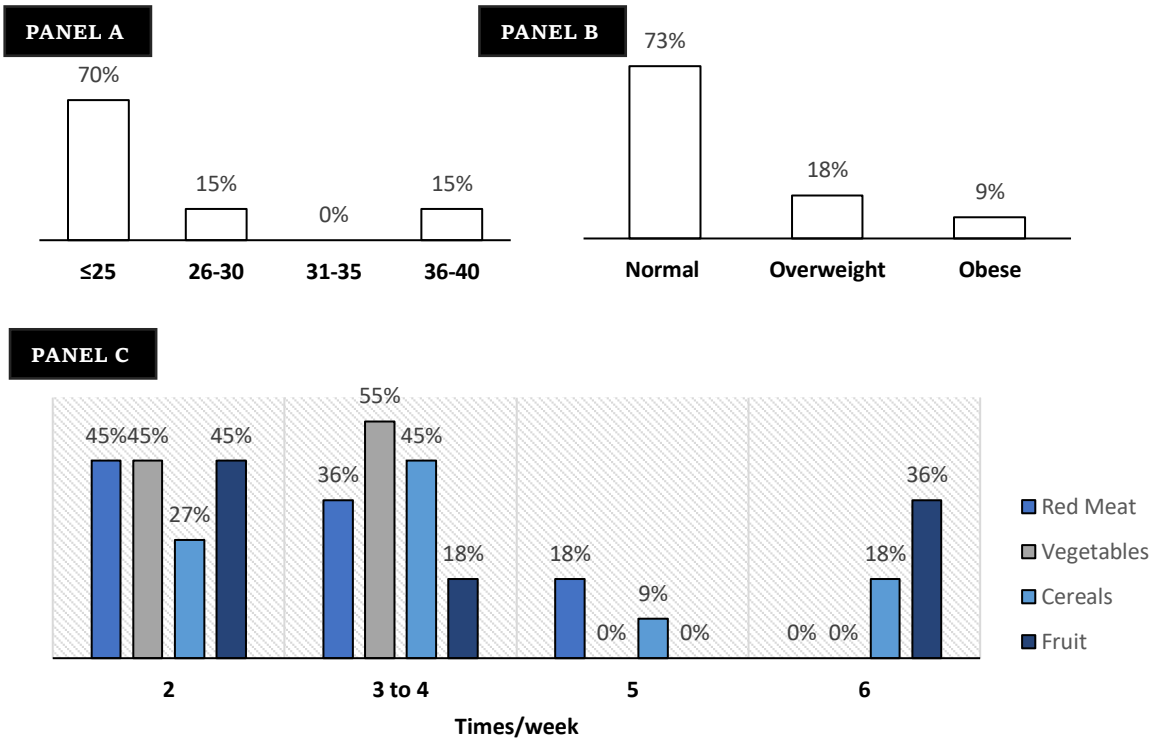


Figure A.8. Age and BMI distribution, and dietary patterns for population B. *Panel A – Age distribution; Panel B – BMI distribution; Panel C – Dietary habits.*

2.2 Semen Analysis

Over two-thirds (69%) of the samples showed values above the WHO cut-off threshold (normozoospermia). Teratozoospermia (23%) and oligozoospermia (8%) were the only abnormalities found (**Table A.9**). **Table A.10** provides a comprehensive summary of the mean (and range) results for the main sperm parameters in analysis. The average abstinence period for semen donors was 2.7 days and 2.4 ml for the ejaculate. When comparing the semen parameters studied with the thresholds defined by the WHO[30], all values showed averages well above the threshold. In particular, the values were 1 to 3 times higher for most parameters, except for morphology, which had a modest increase of only 7,5% in the percentage of typical forms found. The mean PR motility was 63%, and the average total motility was 72.7%. The samples exhibited an average concentration of 46 million sperm cells/ml, resulting in 110.6 million sperm cells per ejaculate. Morphology, on the other hand, was only slightly above the WHO threshold, averaging 4.3% of typical forms. Lastly, the semen parameters for each male patient of study population B can be found, in detail, in Appendix I. Semen Parameters of Population B.

Table A.9. *Semen profile for population B.*

	Semen Sample Classification			
Frequency	N	O	T	Total
N	9	1	3	13
%	69	8	23	100

N, normozoospermia; O, oligozoospermia; T, teratozoospermia.

Table A.10. *Semen parameters for study population B.*

		Semen Donors			5^{th} perc. WHO	Δ (%)
		Mean	Min	Max		
Abstinence period (days)		2.7	2.0	5.0		
Volume (mL)		2.4	0.8	10.1		
Vitality (%)		81.7	90.0	92.0		
Motility (%)	IM	25.27	15.0	47.0		
	NP	8.47	3.0	15.0		
	PR	63.4	46.0	78.0	32.0	+98
	Total	72.7	53.0	85.0	40.0	+82
Sperm Count (x10^6)	Conc. (10^6/ml)	46.1	5.2	192.3	15.0	+207
	TSC	110.6	6.6	436.8	39.0	+182
	TMSC	80.4	4.4	327.6	-	
	TPMSC	70.1	3.5	288.3	-	
Morphology	% NormaL	4.3	2.0	10.0	4.0	+7.5
	TZI	1.5	1.4	1.7		

Perc., percentile; Min, minimum; Max, maximum; Δ, Variation, in percentage, obtained from the following formula: (pop. A mean- 5^{th} percentile)/5^{th} percentile; IM, immotile; NP, Non-progressive; PR, progressive; TSC, total sperm count; TMSC, total motile sperm count; TPMSC, total progressive motile sperm count; TZI, teratozoospermia index.

2.3 Sperm DNA Fragmentation & Sperm Aneuploidy Test

Of the donated samples, the nine normozoospermic semen samples had an average sDF rate of 23.0% (**Table A.11**). These samples were provided by men with an average age of 28.2 years. The four abnormal semen samples exhibited a higher sDF rate of 40.9% despite belonging to men who were, on average, seven years younger (21.5 years). In general, the calculated sDF for the provided samples was 32.1%. Lastly, SAT was performed on samples numbers 2 and 9. In both samples, only haploid cells were found for the analysed chromosomes. The XY ratio was 1.4 for sample number 2 and 1.2 for sample number 9.

3. The Retrospective Cross-sectional Study (2010 – 2021) of infertile men (population C)

3.1 Self-reported lifestyle behaviours

From January 2010 to September 2021, 888 infertile men (study population C) underwent semen analysis at the CHTMAD andrology lab. Most (86%) suffered from primary infertility. The average age of the individuals included in the study was 34.5 years, with a range between 20 and 65 years (**Table A.12.**). Most of the data (58.6%) consisted of individuals older than 30 years and under 41 years of age (**Figure A.9**).

***Table A.11**. Sperm DNA Fragmentation values for population B*

		sDF (%)	Age (years)
n= 13	**Mean*±SD**	32.1±14.8	25.8±7.0
Sample Classification			
1	N	30	22
2	N	10	24
3	N	4	21
4	N	27.8	40
5	N	52.5	22
6	N	24.4	22
7	N	37.9	40
8	N	32.8	28
9	N	34.5	30
	Mean	23.0	28.2
10	O	28.8	24
11	T	31.3	22
12	T	53	22
13	T	50.8	18
	Mean	40.9	21.5

*Arithmetic mean; SD, standard deviation; N, normozoospermia; O, oligozoospermia; OAT, oligoasthenozoospermia; T, teratozoospermia. Samples shaded grey were subjected to SAT.

Among the participants, 22.9% were under or equal to 30 years old, while 18.5% were over 40. The mean BMI of the participants was 26.36 kg/m^2. Furthermore, most individuals (46.5%) were classified as overweight; a significant proportion (17.4%) fell into the obese category, with most of them being moderately obese (**Figure A.9.**). In conclusion, only 36% of the participants had a healthy weight.

Regarding exercise, 70% of the men reported engaging in regular physical activity. Among them, 39.2% exercised up to two times a week, while 30.8% exercised more than two times a week. Smoking habits were prevalent among 35.3% of the participants, with the majority (18.9%) consuming 10-20 cigarettes daily. Furthermore, 40% of the individuals admitted to consuming alcohol, with the highest proportion (23.1%) reporting very light alcohol consumption (limited to one drink per day) (**Table A.12**).

***Table A.12**. Main characteristics of Population C*

Age (years)	**BMI (kg/m^2)**	**Meals (%)**			**Physical Exercise (%)**		**Smoking Habits (%)**			**Alcohol Consumption (%)**		
		Times/day			Times/week		cigarettes/day			Drinks/day		
		≤2	3-4	≥5	1-2	>2	5-10	10-20	>20	1	2-4	>4
34.5	26.36	6	78	17	39.2	30.8	12.6	18.9	3.8	23.1	14.4	3.4

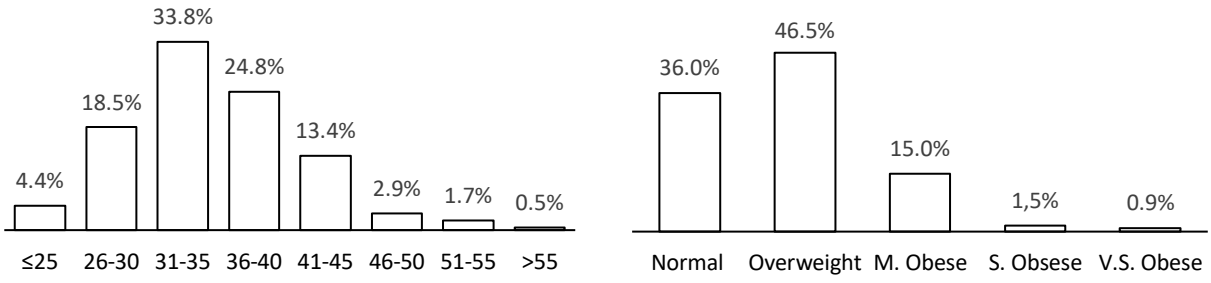


***Figure A.9. Age and BMI distribution for population C.** Panel A – Age Distribution. Panel B – BMI distribution. M. – moderate; S – severe; V.S. – very severe.*

Meal consumption was reported to occur more frequently, 3 to 4 times daily. Cereal consumption was relatively less frequent, while fruit consumption was the highest (35% of the participants reported consuming fruit six or more times a week). Consumption of red meat and vegetables occurred, for the majority, 3 to 4 times a week (**Figure A.10**).

Regarding environmental agents, 41% of the participants reported contact with relevant agents. Specifically, 30% reported contact with paints, 28% with pesticides, and 24% with heat sources. Lastly, among the participants who underwent semen analysis between 2010 and 2021, the majority were service employees (40%), followed by industrial workers (25%), professionals (16%), farmers (7%), technicians (7%), and entrepreneurs (5%). In particular, the five most recurring professions of the more than 80 reported were farmers (7%), drivers (7%), mechanics (5%), construction workers (5%), and police officers (3%) (**Figure A.10**).

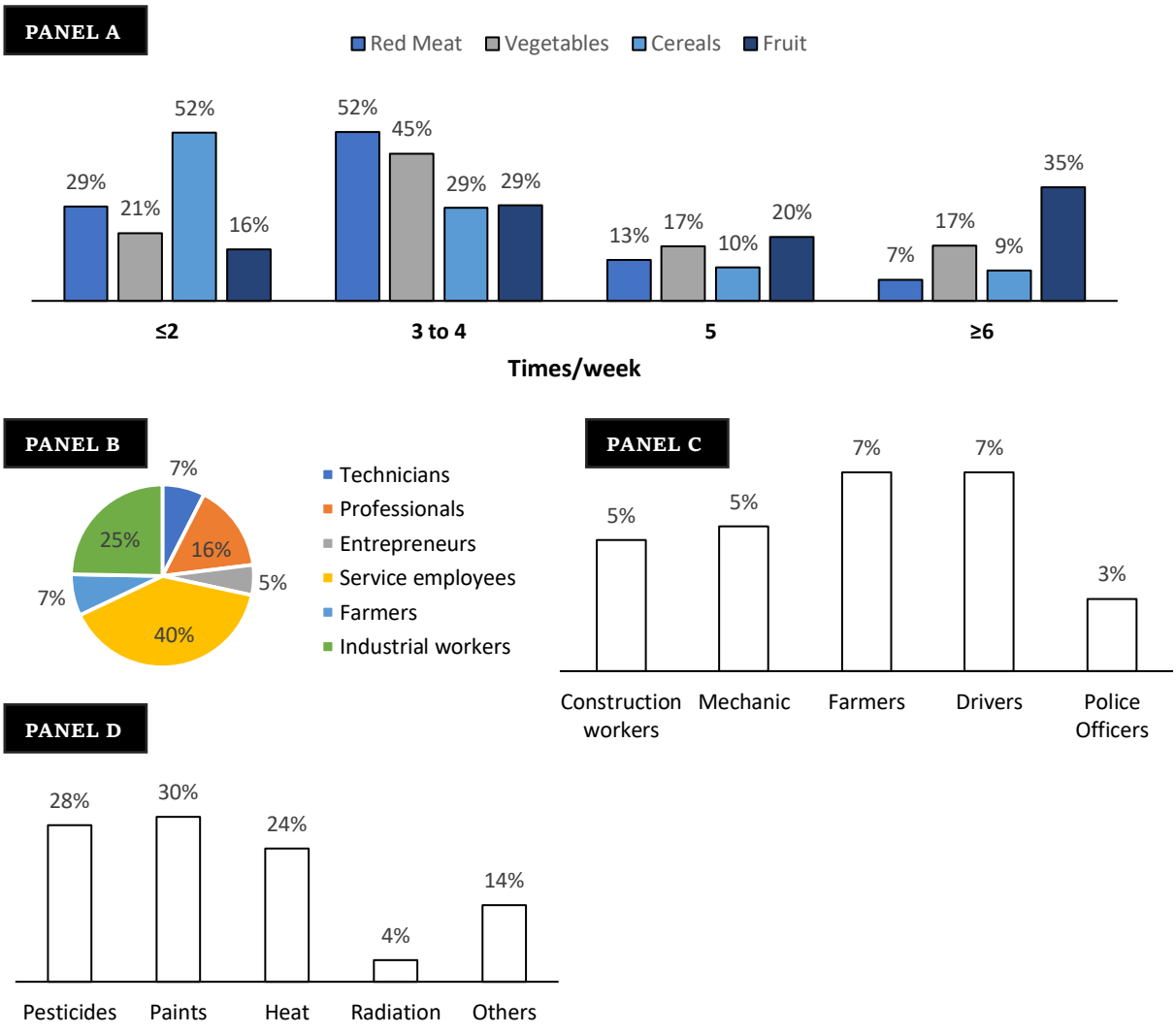


Figure A.10. Lifestyle related factors for population C. *Panel A – Dietary habits. Panel B – Auto reported occupations. Panel C – Top five most reported occupations. Panel D - Reported environmental and occupational exposure.*

3.2 Semen Analysis

Spanning 11 years, 888 semen samples from infertile males (study population C) were subjected to semen analysis. Examination of the gathered reports indicated normozoospermia in more than half of the received semen samples (55.4%), followed by teratozoospermia (19%), oligozoospermia (8.4%), oligoteratozoospermia (4.6%), oligoteratozoospermia (2.9%), azoospermia (2.7%), asthenoteratozoospermia (2.5%), asthenozoospermia (2.4%), oligoasthenozoospermia (1.6%), and finally, criptozoospermia (0.5%) (**Table A.13**). Furthermore, for this population, 53% of the oligozoospermic samples had a sperm count below 5 million sperm cells/ml, and, of these, only 34% had a sperm count greater than 2 million/ml. Lastly, necrozoospermia was found in 34 semen samples (4.1%). Mild necrozoospermia was the most observed anomaly (24 samples), followed by moderate (20-40% vitality) and severe necrozoospermia (below 20% vitality) at 22% and 11%, respectively.

Table A.14 provides a summary of the mean (and range) results for the main semen parameters in analysis. The recorded abstinence period was 3.4 days, with an average ejaculate volume of 2.9 ml. The average progressive motility was 49.3%, while the total motility was 66.5%. The samples showed an average concentration of 48 million sperm cells/ml, resulting in 143.1 million sperm cells per ejaculate and an average of 4.3% typical forms per ejaculate. Compared directly with the WHO threshold, a gain in motility values was observed, for this population, with 54% for progressive motility and 66% for total motility. Regarding sperm count, the values were three to four times higher than the 5th percentile defined by WHO. Morphology exhibited the smallest increase, only 7.5% above the WHO threshold.

Table A.13. *Population C semen profile.*

	Semen Sample Classification										
Frequency	A	AT	AZ	C	N	O	OA	OAT	OT	T	Total
N	21	22	24	4	492	75	14	26	41	169	888
%	2.4	2.5	2.7	0.5	55.4	8.4	1.6	2.9	4.6	19.0	100

A, asthenozoospermia; AT, asthenoteratozoospermia; AZ, azoospermia; C, criptozoospermia; N, normozoospermia; O, oligozoospermia; AO, oligoasthenozoospermia; OAT, oligoasthenoteratozoospermia; OT, oligoteratozoospermia; T, teratozoospermia.

3.3 Cytogenetic and Molecular Studies

From 2010 to 2021, 491 infertile men were referred for cytogenetic analysis. Among the study participants, 13 men were identified as having cytogenetic alterations (**Table A.15**). This accounts for an incidence rate of 2.6% of cytogenetic abnormalities within the studied population. **Table A.15** describes the cytogenetic abnormalities found, their frequency, and the corresponding semen profile. A total of five reciprocal translocations were identified, exclusively involving autosomes, and one case of a Robertsonian translocation between acrocentric chromosomes 13 and 14. Furthermore, aneuploidies were observed in seven individuals, one with 47,XYY (Jacobs syndrome) and six with 47,XXY (Klinefelter syndrome). Despite limited data, most cytogenetic anomalies were accompanied by an altered semen profile. On the other hand, YCMD studies carried out in a cohort of 27 men did not detect AZF deletions.

***Table A.14**. Semen parameters for population C*

		Infertile Males (2010-2021)			5th perc. WHO	Δ (%)
		Mean	Min	Max		
Abstinence period (days)		3.4	2.0	5.0		
Volume (mL)		2.9	0.1	9.6		
Vitality (%)		73.2	8.0	97.0		
Motility (%)	IM	27.5	4.0	97.0		
	NP	12.1	2.0	52.0		
	PR	49.3	0.0	95.5	32.0	+54
	Total	66.5	3.0	96.0	40.0	+66
Sperm Count (x10^6)	Conc. (10^6/ml)	48.4	2.1	516.3	15.0	+222
	TSC	143.1	2.4	1394.0	39.0	+267
	TMSC	95.1	0.6	1179.0	-	
	TPMSC	70.8	0.0	1018.3	-	
Morphology	% Normal	4.3	0.0	71.0	4.0	+7.5
	TZI	1.6	1.2	2.5		

Perc., percentile; Min, minimum; Max, maximum; Δ, variation, in percentage, obtained from the following formula: (pop. A mean - 5th percentile)/5th percentile. IM, immotile; NP, non-progressive; PR, progressive; TSC, total sperm count; TMSC, total motile sperm count; TPMSC, total progressive motile sperm count; TZI, teratozoospermia index.

***Table A.15**. Cytogenetic findings in male infertile patients from 2010 to 2021 and the respective semen profile.*

	Karyotype	Frequency (n)	Semen Profile
Robertsonian Translocations	45,XY,der(13;14)(q10;q10)	1	Oligozoospermia
Reciprocal Translocations	46,XY,t(4;10) (q31.3;p15)	1	Oligoteratozoospermia
	46,XY,t(4;22)(p16.1;q11)	1	Oligoasthenozoospermia
	46,XY,t(6;8)(p23;p21.3)	1	Normozoospermia
	46,XY,t(8;17)(p22;q21.32)	1	NA
	46,XY,t(11;22)(q14.2;q13.1)	1	Oligoteratozoospermia
Aneuploidies	47,XYY	1	NA
	47,XXY	6	Azoospermia

NA – no available data

4. Comparative Analysis

4.1 Lifestyle

4.1.1 Age & BMI

Notable differences in the age composition among the populations were found and summarised in **Figure A.11**. Populations A and C show a relatively balanced distribution across different age groups. In contrast, population B is dominated by individuals under the age of 30 years. Both populations derived from fertility consultations (A and C) have a relatively higher representation in the 31-40 age group, with population A accounting for 63% and population C accounting for 58.6% of individuals in this age group. Additionally, a declining representation in older age groups is also seen: population A shows a gradual decrease from the 36-40 age group onwards, and population C exhibits a decreasing proportion from the 31-35 age group onwards.

A comparison of weight categories among the three populations (A, B, and C) revealed different characteristics (**Figure A.11**). Although populations A and C showed similar patterns, with a higher prevalence of overweight (47 and 46.6%, respectively) and obese individuals (16% and 7.4%, respectively), population B showed a higher proportion of individuals with a healthy weight (73%).

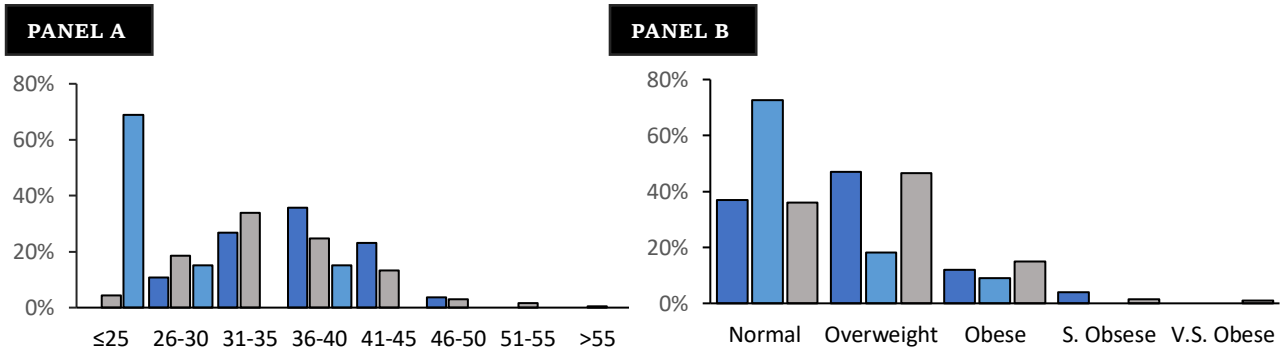


Figure A.11. Age and BMI distribution for populations A-C. *Panel A – Age distribution. Panel B – BMI distribution. S – severe; V.S. – very severe. Blue – population A, light blue – population B, and grey – population C.*

4.1.2 Eating Habits

The comparison of the dietary habits of the three populations, even though most report eating 3 to 4 meals a day, indicates some crucial differences. Population A tends to consume more red meat (22% vs 18% and 19% of individuals who consume red meat at least five times a week, for populations B and C, respectively) and vegetables (44% vs 0% and 34%). In comparison, population B has a higher frequency of cereal consumption (27% vs 19% and 20% for populations A and C, respectively). Lastly, population C reports the highest frequency of fruit consumption (55% vs 51% and 36% for populations A and B, respectively) (**Figure A.12**).

Regarding the category of food least consumed, for population A, it consisted of cereals, with only 19% reporting a frequency of at least five times a week; for population B, it was vegetables, with no individuals reporting a frequency of at least five times a week. Lastly, red meat and cereals were the least consumed by population C. The reported frequency of consumption category was only 19% and 20%, respectively, for the at least five times per week category.

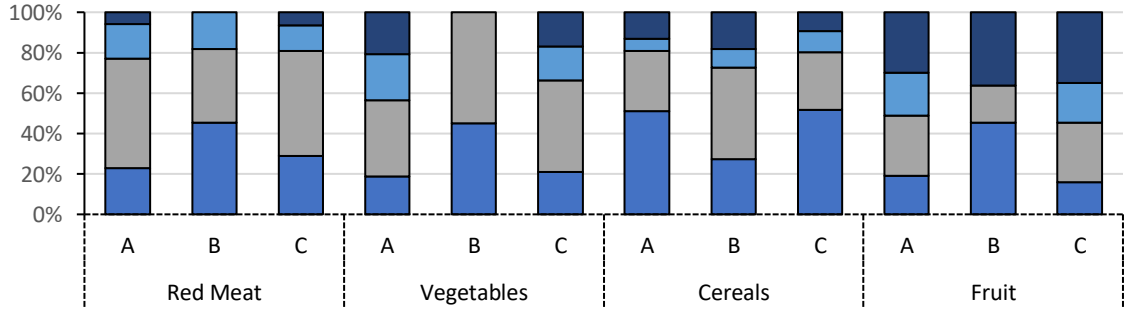


Figure A.12. Dietary habits for the populations in study. *Blue - ≤2 times/week; grey – 3 to 4 times/week; light blue – 5 times/week; dark blue - ≥ 6 times/week.*

4.1.3 Exercise, Smoking, and Alcohol Consumption

Figure A.13 provides information on the frequency of physical activity between populations A, B, and C. Population B shows a higher proportion of men who engage in physical activity more than two times a week (64%). On the contrary, populations A and C display similar patterns, with the highest percentage of individuals exercising only twice weekly (45% and 39%, respectively). Overall, the three populations studied show a similar share of individuals who report not engaging in physical activity (27% for population B and 30% for populations A and C).

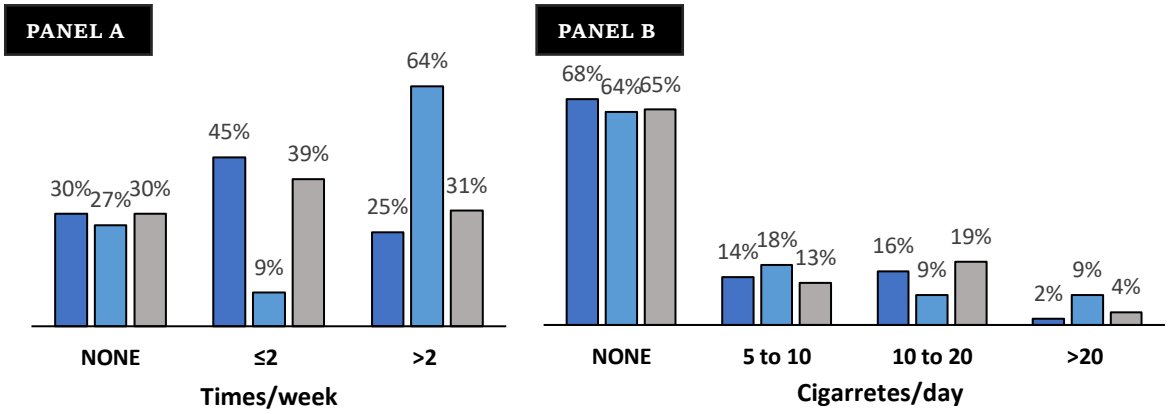


***Figure A.13. Frequency of physical exercise and smoking**. Panel A – Frequency of physical activity. Panel B- Frequency of smokers. Blue – population A, light blue – population B, and grey – population C.*

Regarding smoking habits among the three populations, most reported not smoking (68%, 64%, and 65% for populations A, B, and C, respectively). Furthermore, there is a consistent pattern between populations A and C, where a higher percentage of men reported smoking 10 to 20 cigarettes daily (16 and 19%, respectively). For population B, the highest proportion of men only smoke 5-10 cigarettes/day (18%). In addition, the highest frequency of individuals smoking more than 20 cigarettes per day can also be found in this population.

Most of the individuals in each population reported no daily alcohol consumption (66, 55, and 59% for populations A, B, and C, respectively) (**Figure A.14**). Among those who reported drinking, the most common category in the three populations was the consumption of one drink per day. However, population B shows the highest percentage of individuals in this category (27%). Population C has the highest percentage of men who reported consuming 2 to 4 daily drinks (14%). In contrast, populations A and B share the highest percentage of individuals who report consuming more than four drinks per day (9%).

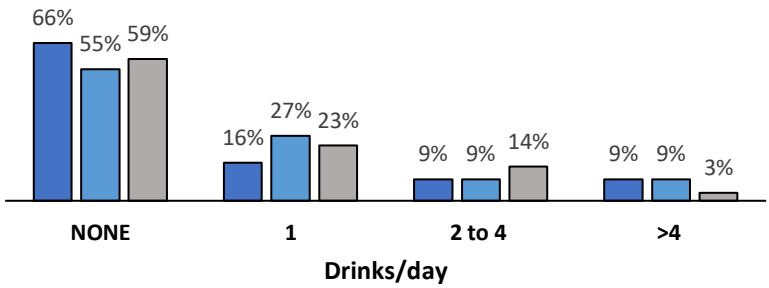


***Figure A.14. Alcohol consumption.** Blue – population A, light blue – population B, and grey – population C.*

4.1.4 Environmental agents and occupation

Slight differences in reported exposure to the listed agents were found between populations A and C (**Figure A.15**). Population C shows a higher percentage of individuals who report exposure to pesticides and heat sources (11% and 10%, respectively) compared to population A (7% for both agents). On the other hand, population A exhibits a higher percentage of individuals reporting radiation exposure (4% vs 2%). Most in both populations reported no exposure to any agent (71% and 59%, population A and C, respectively). Among those who reported exposure, the most reported agents in both populations were paints (14% and 12%, population A and C, respectively), followed by pesticides (7% and 11%) and heat sources (7% and 10%).

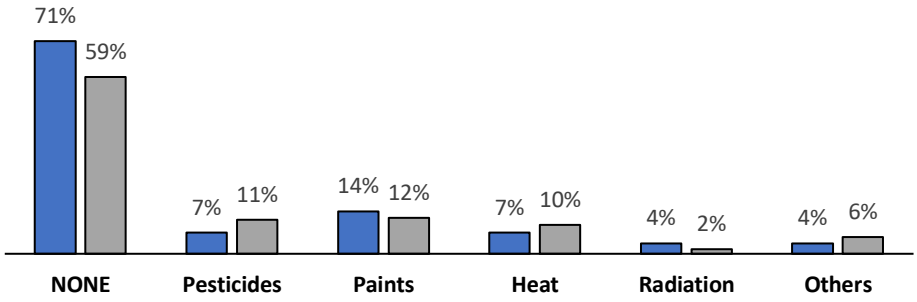


Figure A.15. Exposure to environmental and occupational agents. *Blue – Population A; grey – population C.*

A comparison of the occupational distribution of populations A and C can be found in **Figure A.16**. Both populations are comprised of a significant proportion of service employees (31% and 40%, respectively) and industrial workers (23% and 25%, respectively). Additionally, entrepreneurs/businessmen constitute the smallest workforce share in both populations, although slightly higher in population C (3% vs 5%).

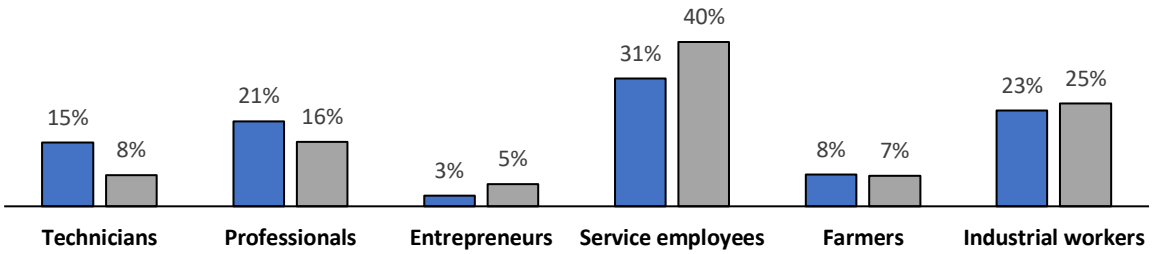


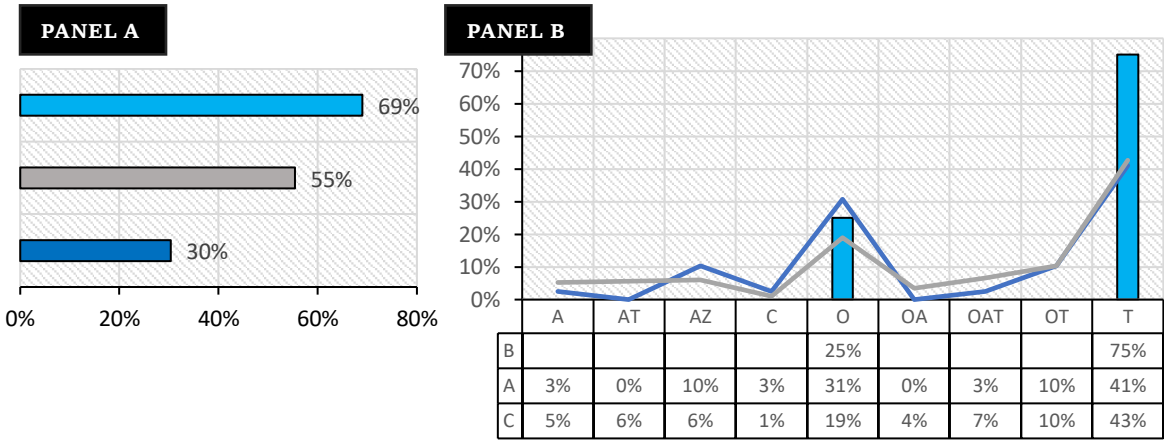
Figure A.16. Auto-reported occupations for populations A and C.

4.2 Semen analysis

4.2.1 Semen profile

Population B shows the highest percentage of normozoospermic samples (69%) and population A the lowest (30%) (**Figure A.17**). Regarding anomalies in semen evaluation, population B only showed samples with oligozoospermia (25%) and teratozoospermia (75%). Population A displayed the highest frequency of oligozoospermic (31%) and azoospermic (10%) samples. Other abnormalities in semen for this study population were as follows: 5% asthenozoospermia, 3% criptozoospermia, and oligoasthenoteratozoospermia each, 10% oligoteratozoospermia, and 41% teratozoospermia. Population C had 5% asthenozoospermic samples, 6% asthenoteratozoospermic and azoospermic, each, 1% criptozoospermic, 19%

oligozoospermic, 4% oligoasthenozoospermic, 7% oligoasthenoteratozoospermic, 10% oligoteratozoospermic, and 43% teratozoospermic samples.



	A	AT	AZ	C	O	OA	OAT	OT	T
B					25%				75%
A	3%	0%	10%	3%	31%	0%	3%	10%	41%
C	5%	6%	6%	1%	19%	4%	7%	10%	43%

Figure A.17. Results of semen analysis at CHTMAD and percentage of abnormalities found. Panel A - Percentage of normozoospermia. Panel B – Frequency of semen abnormalities. Blue – population A; Light blue – Semen donors; Grey - population C; A- asthenozoospermia; AT – asthenoteratozoospermia; AZ – azoospermia; C – criptozoospermia; O – oligozoospermia; AO – oligoasthenozoospermia; OAT – Oligoasthenoteratozoospermia; OT – oligoteratozoospermia; T – teratozoospermia.

4.2.2 Motility

When comparing the motility averages of the different study populations with the 50th percentile values of fertile men according to the WHO [30], it was observed that population B exhibited higher levels of progressive and total motility (8% and 11% difference, respectively) (**Figure A.18**). On the other hand, in male infertile patients from populations A and C, the mean total motility was above the described values for fertile patients. However, progressive motility was slightly lower (3 to 6%).

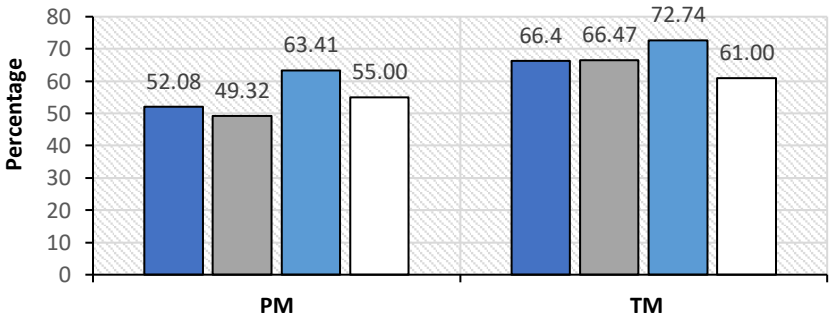


***Figure A.18. Progressive (PM) and total motility (TM) for infertile patients and semen donors**. Blue – Population A; Grey – population C; Light blue – population B; White – 50th percentile for fertile men, as described by WHO (2010).*

4.2.3 Vitality

Donors (population B) represent the group with the highest recorded vitality, even exceeding the 50th percentile of fertile men provided by the WHO (**Figure A.19**). To no surprise, male infertile CHTMAD patients (population A and C) had, on average, a lower vitality. However, a more in-depth analysis reveals the real drivers behind these observations. Since population B showed higher total motility, the vitality numbers, as a consequence, are understandably

higher. Indeed, when looking at the percentage of live immotile sperm (% vitality - % total motile sperm), a very different scenario is observed. The fertile men's group exhibits the highest percentage of live immotile sperm (18%). In contrast, the study population of infertile male patients (populations A and C) and semen donors (population B) show a much lower percentage of live immotile sperm cells.

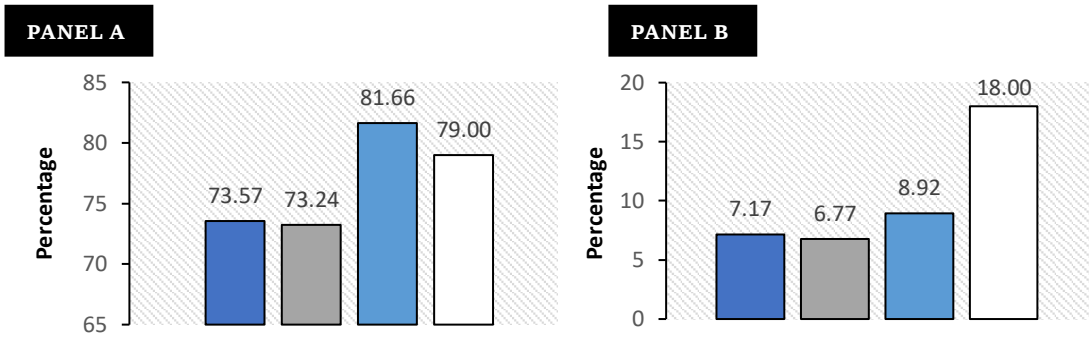


Figure A.19. Vitality. *Panel A – Percentage of vitality by group study. Panel B – Percentage of live immotile spermatozoa, by group. Blue – population A; Light blue – population B; Grey – population C; White – 50th percentile for fertile men (WHO, 2010).*

4.2.4 Sperm count

On average, the three populations studied exhibited a TSC that was above the lower reference limit but below the 50th percentile for fertile men (**Figure A.20**). However, particular attention should be paid to infertile men from population A as they have the lowest average sperm concentration and TSC among the groups. In contrast, infertile males in population C demonstrate the highest average values of sperm concentration (per ml, they show more 18 million sperm cells than population A) and TSC (more 55.28 million sperm cells per ejaculate than population A). Interestingly, semen donors from population B exhibited only a 31.5% increase in TSC compared to A. Additionally, population A shows the highest prevalence of severe oligozoospermic (less than 5 million sperm cells/ml) samples, accounting for 53% of the cases. Within this group, 88% of the samples have a sperm concentration below 2 million sperm cells/ml (**Figure A.21**).

Another way to express sperm quality is through the calculation of total motile sperm count (TMSC) or even total progressive motile sperm count (TPMSC), obtained by multiplying the volume of the ejaculate (in ml) by the sperm concentration and the proportion of motile or progressive motile sperm cells, respectively. Regarding our study population, population A again showed the lowest values for the parameters in discussion (58.53 and 45.76 million sperm cells for TMSC and TPMSC, respectively). Population B, in contrast, was rescued by its high levels of progressive motility (63.41%) and showed almost identical TPMSC (70.13) as population C (70.76) but overall lower TMSC (80.44 vs 95.13 million sperm cells). The 50th percentile for fertile males shows values for TPMSC and TMSC, 63% and 41% higher than the ones for population C.

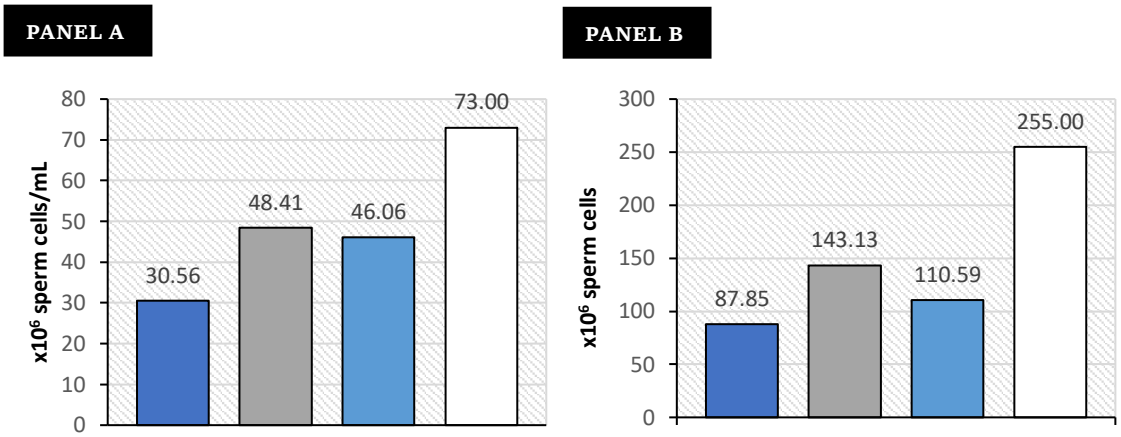


Figure A.20. Sperm concentration and total sperm count by study groups. *Panel A – sperm concentration. Panel B – Total sperm count. Blue – population A; Grey – population C; Light blue – population B; White - 50th percentile for fertile men (WHO, 2010).*

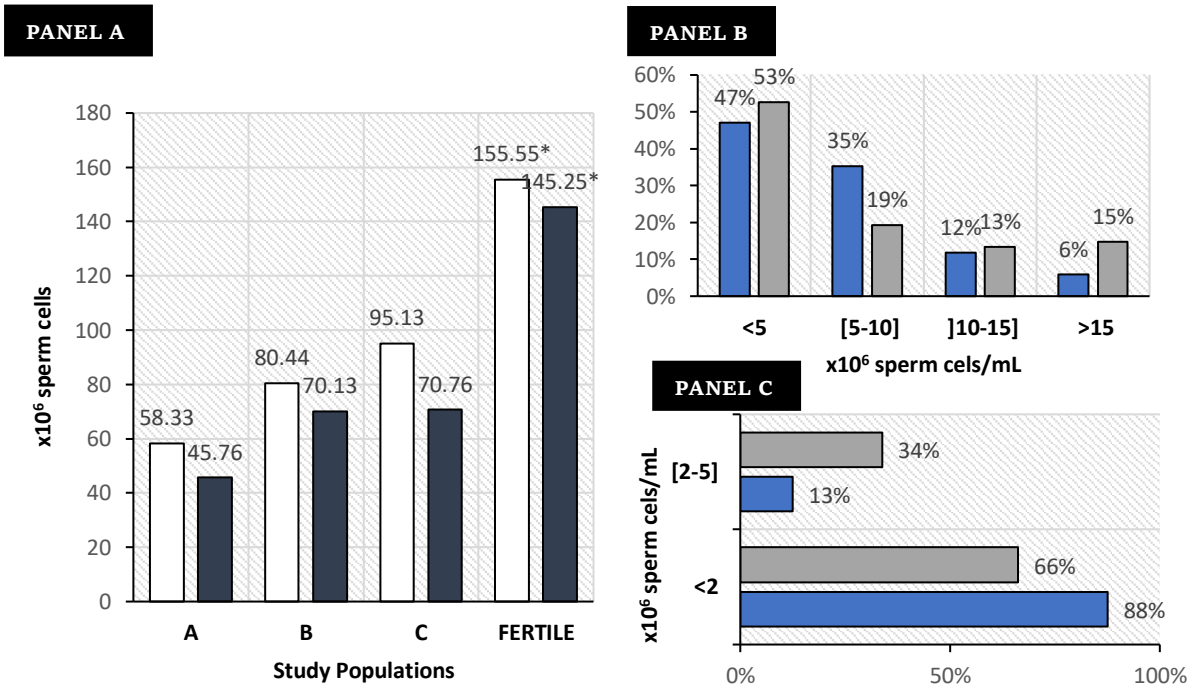


Figure A.21. Sperm count and oligozoospermia*. Panel A – Total motile sperm count (TMSC), white, and total progressive motile sperm count (TPMSC), blue, by study groups. Panel B – Sperm concentration of men classified as oligozoospermic, and respective frequency. Panel C. Sperm concentration of infertile males displaying severe oligozoospermia, and their respective frequency. Blue – population A; Grey – population C.* - Estimation based on the reported values of Total Sperm Count, Progressive Motility and Total Motility for fertile population (50th percentile) by WHO [30].*

4.2.5 Morphology

Population A shows the lowest frequency of normal forms per ejaculate, while the remaining analysed population displayed a frequency of 4.31% typical forms. The fertile population exhibits, on average, four times the frequency of typical forms. Regarding the total normal sperm count (TNSC), defined as the product of the total sperm count and the percentage of normal morphology (total sperm concentration x (%) normal morphology/100), the disparities between the fertile group and the remaining groups are further exacerbated. The fertile group exhibits almost six times the TNSC obtained for population C (the best-performing group out of the three) (**Figure A.22**).

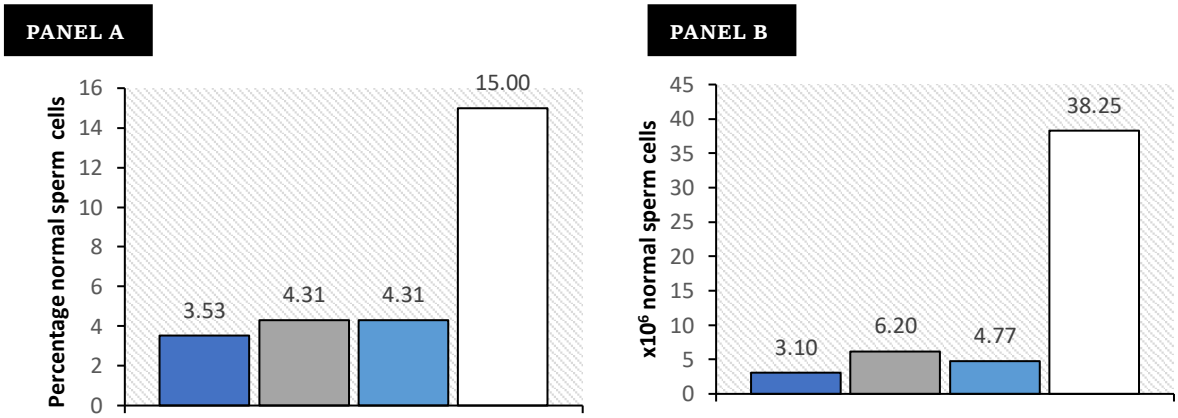


Figure A.22. Morphology Assessment. *Panel A- percentage of typical forms, on average, by study groups. Panel B- Total Normal Sperm Count (TNSC), by groups. Blue – population A; Grey – population C; Light blue – population B; White - 50th percentile for fertile men [30]. TNSC was calculated according to the following formula: (% normal forms) x (total sperm count).*

4.3 DNA Damage

Significant disparities in DNA damage levels were observed between populations A and B (**Figure A.23**). Population A exhibited, on average, a higher percentage of sperm DNA damage (65.9%), accompanied by higher rates of DNA fragmentation in both the normozoospermic (67%) and abnormal semen samples (64.2%). In contrast, population B showed lower overall DNA damage (32.1%), characterised by a comparatively lower DNA damage rate in the normozoospermic subgroup but a higher rate in the abnormal subgroup (23.0% vs 40.9%).

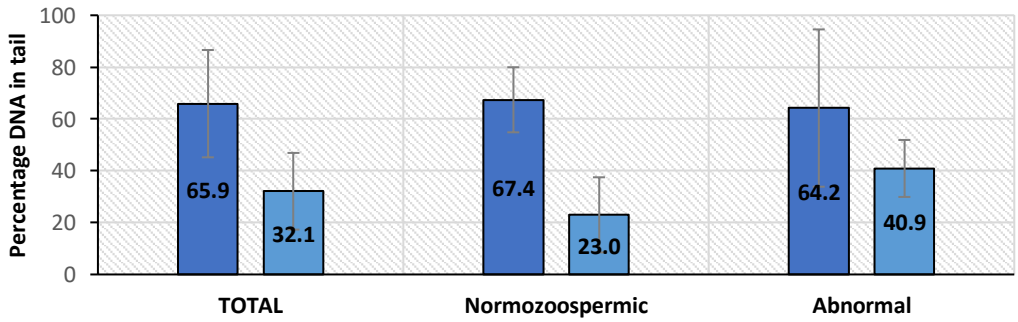


Figure A.23. Sperm DNA fragmentation for study population A (blue) and B (light blue). *Bars represent standard deviation.*

V. DISCUSSION

The average number of births per woman in Portugal in 2021 was 1.34, representing a significant drop compared to the fertility rate of 3.20 in 1960 [108]. The first national epidemiologic study on infertility estimates that up to 290,000 couples are infertile, and up to 61% seek medical assistance [109]. Furthermore, due to the higher population density, most infertile couples are found in Lisbon/Vale do Tejo and the north region.

For the CHTMAD region of reference, there are no reliable epidemiological data for infertility. However, essential trends are still evident. In particular, the low level of fertility is due to the growing proportion of women giving birth later in life. In fact, in 2014, 56% of all recorded childbirths at the CHTMAD were from women aged 30 years or older. By 2020, this share had increased to 64% [57]. At a national level, the age at which women gave birth was 30.9 years in 2021, with women in the northern region of Portugal reaching, on average, 31.2 years at birth [108].

1. Infertility, Lifestyle & Reproductive behaviours

1.1 Paternal Age

Temporal patterns of reproductive behaviour have suffered drastic changes over the years. Parenthood often competes with education and employment aspirations, and with the introduction of reliable contraceptive methods, parenthood has become an issue of personal preference. The result has been a massive increase in childbearing age for both sexes. This postponement is further exacerbated by low gender equity, changes in partnership behaviour, limited housing availability, and economic uncertainty [110].

According to a survey conducted in 2019 [111], in Portugal, 97.6% of men aged 18 to 29 years had no children, and more than half of men between 30 and 39 years remained childless. There is no clear distinction between voluntary and involuntary childless in these numbers, but the trend is clear. Consequently, a large majority of men (89% in population A and 71% in population C) who were referred for semen analysis during fertility investigations were over the age of 30 years (**Figure A.11**). Although younger men are less likely to seek fertility consultations due to their partners' high fertility, the awareness of potential reproductive dysfunctions arises only when there is a strong desire to start a family. As the desire to have children has been increasingly expressed later in life, there is a higher representation of men over 30 in fertility consultations. This suggests that the delay in family planning is responsible for the observed over-representation of older men seeking fertility assistance in populations A and C. Population B is composed mainly of students and, therefore, of much younger individuals.

Fertility clinics and reproduction centres face the fact that an increasing proportion of men seek assistance when their partner's fertility is already in decline [112]. That is, any suboptimal reproductive function in the male will most likely not be compensated for by their female partner.

1.2 Lifestyle

1.2.1 Eating Habits

Recommendations from the WHO advocate for adults the daily intake of 5 servings of fruits and vegetables (35 servings/week), 4.5 servings (31.5 servings/week) or 180 g of grains/cereals, while red meat should be eaten 1–2 times per week [113]. Furthermore, the Portuguese Association of Nutritionists (APN) recommends 5 to 6 daily meals [114]. Given

this, the males studied exhibit poor nutritional habits with low cereal, vegetables and fruit intake and levels of red meat consumption above the recommended. In particular, among infertile male patients, populations A and C reported a frequency of red meat consumption greater than two times a week. This dietary habit was observed in 77% of the cases for population A and 71% for population C. For population B, only 55% report a red meat intake above the WHO recommendation. Furthermore, the frequency of meals in the three populations studied is severely below the APN recommendation. Only 15%, 9%, and 17% of the individuals in populations A, B, and C, respectively, met the recommended five meals or more daily.

Interpreting data on vegetable, fruit, and grain/cereal intake is challenging due to the less-than-ideal conversion of WHO recommendations from servings to times per week. Such imprecision is further exacerbated by the "6 times or more/week" category in the questionnaire. This makes it difficult to assess adherence to the recommended intake accurately. However, based on available data, it can be observed that a significant proportion of individuals in the three populations (A, B, and C) do not meet their recommended daily intake of vegetables (57%, 100%, 66%), cereals (81%, 72%, 81%) and fruit (49%, 63%, 45%) in a given week. The National Food, Nutrition, and Physical Activity Survey conducted in 2017 [114] upholds these findings, underscoring that Portuguese men, in particular, tend to have a higher and lower frequency of meat, and fruit/vegetable consumption, respectively.

1.2.2 BMI & Physical Activity

High-fat diets are known to affect reproduction [115]. As found by Rato et al. [116], testicular metabolism is altered by high-energy diet intake and obesity. According to the World Obesity Federation [117], nearly 60% of the male Portuguese population is obese or pre-obese. When broken down by age, the data show that this share increases with age. The lowest percentage (31.9%) is observed in men aged 18-24, while men aged 35-44 exhibit a higher share of obesity at 61%. The prevalence continues to increase, with the highest percentage observed in men aged 65-74, peaking at 73.3%. Consequently, our data, once again, closely resemble the findings for the general/unselected (Portuguese) male population: for population B, the youngest demographic, 27% of individuals are obese or pre-obese; for populations A and C, where the average male age is 36.7, and 34.5, respectively, this share reaches 63-64%.

Semen donors in population B were of a younger demographic age, had a healthier weight, and had a higher frequency of regular exercise compared to male patients seeking fertility consultations at CHTMAD. Studies found an inverse association between age, civil status, and frequency of physical activity [118]. Single young adults are the most physically active adults, and since they comprise a large portion of population B, the observed trends are in agreement with the literature. In addition, epidemiological studies consistently find that subjects with a higher BMI are more prone to physical inactivity [119]. Therefore, and as echoed by the recruited semen donors, Portuguese males aged 18-30 are the most physically active adults and, consequently, the least overweight. Interestingly, despite having a higher BMI, male patients subjected to fertility testing reported nearly the same level of physical activity as semen donors (73% vs 70% for both populations A and C). This difference may be due to the auto-reported frequency of physical activity. Only 25% to 31% of the male patients (population A and C, respectively) report being physically active more than two times a week, while for population B individuals, this share reaches 64%.

1.2.3 Smoking and Alcohol Consumption

According to data from the 2019 National Health Survey [118], 23.9% of Portuguese males are daily smokers. The highest prevalence of smokers is found in the 25-34 age group (27.6%), followed by the 35-44 age group (24.9%). Despite the different age groups in conflict, the three populations studied display a higher percentage of daily smokers (32% for population

A, 36% for population B, and 35% for population C). Furthermore, according to data from the National Health Survey [118], most male smokers consume 11 to 20 cigarettes per day. This trend is also reflected in populations A and C, where 50% and 52% of individuals report smoking within this range.

Data compiled by Eurostat [120] show that the share of people drinking alcohol daily was highest in Portugal (20.7%), with men consuming alcohol more frequently than women – rates almost four times higher (33.4 vs 9.7%). For population A, a very similar proportion of males (34%) report daily drinking. In contrast, a higher frequency of daily drinking is reported (45% and 41%) for populations B and C.

1.2.4 Environmental & Occupational Hazards

Many studies have shed light on occupational hazards to male reproductive function. However, occupational (and environmental) exposure conditions are complex: first, men in different job descriptions may be exposed to the same agents; second, the same job description may, in reality, be used to describe an array of (distinct) activities with very different implications exposure-wise to potential toxins; third, the existence of multiple confounding variables such as age, smoking, alcohol consumption, diet, socioeconomic status; fourth, it is a cumbersome, nearly impossible task, trying to pinpoint specific toxin exposures in an occupational setting [121, 122]. Unfortunately, for population A, the limited sample size gives no margin for accurate data interpretation and is aggravated by the nonspecific nature of job classifications. Of 56 males, 17 failed to describe their occupation, and the remaining 39 offered 29 distinct (sometimes vague) job descriptions. For population C, the most frequently reported occupations were farmers, drivers, mechanics, construction workers and police officers. According to the scientific literature, the former four occupations are linked to decreased semen quality, lower pregnancy rates, and miscarriages [121, 123].

Environmental and occupation-related fertility factors are mainly due to physical (heat and radiation) and toxic (chemicals) sources. In particular, the National Institute for Occupational Safety and Health (NIOSH) found that more than 1,000 chemicals used in the workplace have been associated with reproductive side effects in animals [124]. Chemical exposure during work can occur in significant ways. Direct contact with heavy metals, commonly found in occupations such as mining, metalworking, and painting, is a frequent source of exposure. Additionally, individuals working in the oil industry and handling industrial solvents, such as paints, varnishes, lacquers, adhesives, glues, degreasers, and cleaning agents, are at risk. Pesticides, often associated with agricultural work, pose a potential hazard [121, 123]. Furthermore, men working in environments with high levels of air pollution may face chemical exposure. Occupations such as tool booth workers and police officers are particularly susceptible to such risks [123]. Increased heat exposure, which results in elevated levels of scrotal temperature (with prolonged sitting), is associated with lower semen quality [122]. For example, professional drivers, computer programmers, chefs, bakers, and ceramic oven operators comprise the group of occupations linked to increased heat exposure [123]. Interestingly, the occupations most commonly reported among infertile male patients at CHTMAD align with occupations that scientific research has already suggested as susceptible to reproductive dysfunction. This further underscores the need for a more comprehensive study investigating the potential effects of occupation and semen quality in infertile males at CHTMAD.

Additionally, exposure to paints and pesticides were the most common toxic agents to which infertile males at CHTMAD (populations A and C) report exposure. For paints, the presence of lead is the main culprit in reproductive dysfunction. Lead disrupts the HPG axis, alters sperm quality, and may decrease overall fertility [125]. Conversely, pesticides negatively affect men's reproductive health by mimicking natural hormones (altering regular hormone

activity) and through their direct toxic effect on sperm cells [125]. However, most infertile males in this study do not report exposure to any relevant chemical or physical agent. Such an observation primarily hints at the surveyed males not being aware of the potential reproductive hazards in their workplace.

In summary, the dietary patterns encountered in the infertile male population studied, and the cohort of semen donors closely resemble the findings for the general/unselected (Portuguese) male population. Interestingly, age emerges as a crucial factor in shaping lifestyle trends, encompassing aspects such as physical activity, diet, smoking, and drinking habits. Additionally, exploring the impact of environmental and occupational factors on male reproductive health in the Trás-os-Montes and Alto Douro region holds significant potential for gaining valuable insights.

2. Semen Analysis

Semen parameters were evaluated in three distinct populations: semen donors (population B), mainly composed of young males residing in Vila Real; infertile male patients who underwent semen analysis at CHTMAD between September 2021 and October 2022 (population A); and a separate group (population C) of infertile male patients who underwent semen analysis from 2010 to 2021. These groups were compared with the 50th and 5th percentile values for fertile men provided by the WHO [30].

For the three studied populations, teratozoospermia was the most common semen abnormality. **Table A.16** resumes the numerous findings of the semen profile of infertile males in different countries. Great variation in the percentage of abnormalities found in each study is apparent. Sample size, selection bias, study design and reporting, age, environmental, cultural, and socioeconomic factors may be responsible for the substantial geographical variation observed for semen parameters [35].

Regarding the present study, it should be noted that two populations (B and C) exhibited mean semen parameters all above the 5th percentile threshold established by the WHO. In contrast, population A failed to do so: the mean percentage of typical sperm cells fell below 4%. As expected, the fertile group represented by the WHO 50th percentile demonstrated better overall semen quality. However, it is interesting that all three populations performed similarly regarding sperm motility, showing values above the average reported for fertile men.

To understand the parameters obtained for each study group and the extent of their variation, it is essential to consider the specific context of the populations under investigation.

Table A.16. *Geographical variation of the semen parameters. Adapted from Tilahun et al.* [126].

	N	**PI**	**A**	**AT**	**AZ**	**C**	**N**	**O**	**OA**	**OAT**	**OT**	**T**
		(%)										
Nigeria	661	30	11.5	0.9	6.2	-	68.2	25.5	2.3	2.1	3.2	18.5
Indonesia	1,186	88.7	1.9	0.9	8.1	0.3	67	13	5.9	1.9	0.1	0.9
Nepal	520	78.5	17	-	12.7	-	56	3.8	7.9	1.5	-	0.8

PI, primary infertility; A, asthenozoospermia; AT, asthenoteratozoospermia; AZ, azoospermia; C, criptozoospermia; O, oligozoospermia; AO, oligoasthenozoospermia; OAT, oligoasthenoteratozoospermia; OT, oligoteratozoospermia; T, teratozoospermia.

2.1 Semen donors and infertile male patients.

For the semen parameters analysed, semen donors and infertile male patients, particularly in population C, did not show a major disparity in values. The latter is even overperforming in parameters such as concentration and TMSC. At first glance, this observation may seem peculiar, considering the contrasting characteristics of the study cohorts: a young and healthy-weight male population, as semen donors, versus older and overweight males experiencing infertility. Furthermore, despite this similarity in sperm parameters, very distinct semen profiles were obtained. Population B showed the highest percentage of normozoospermic samples (69%), followed by populations C (55%) and A (30%). This puzzling observation highlights the inherent challenges of infertility studies, male fertility assessment, as well as the complexities of semen analysis.

WHO has established cut-off values to differentiate between normal and abnormal semen. They also recommend using descriptive nomenclature (to categorise the different forms of male factor infertility). However, studies have found a poor predictive value of the WHO semen analysis classification system for the couple's prognosis and treatment choice [127]. In fact, since T(P)MSC factors in three pivotal parameters in semen analysis (volume, concentration and (progressive) motility), it is considered to be a better indicator of male fertility and predictor of fertility outcomes than the WHO sperm classification system (studies found that TPMSC correlates better with spontaneous pregnancy [127–129]). Interestingly, population C, consisting of infertile males, showed a TPMSC comparable to that of semen donors, with both groups exhibiting around 70 million progressive motile sperm cells per ejaculate (**Figure A.21**).

In addition, several factors may explain why a cohort of infertile patients can exhibit sperm parameters comparable to a randomly selected group of males from the general population. First, the reproductive health of semen donors is unknown since they have never expressed a desire for progeny. Therefore, it is possible that infertility is already present but is not recognised in this group. Second, epidemiologic studies indicate that male factors contribute to up to 50% of infertility cases [33]. This means that men without fertility issues often appear in fertility consultations and undergo fertility tests due to the poor reproductive health of their partners. Third, some studies suggest that using a time frame of less than five years can result in the misclassification of fertile couples as infertile [131]. Research shows that up to 23% of supposedly 'infertile' couples conceive naturally after two years without medical intervention [130]. Fourth, the effect of sample size cannot be ignored, as it can lead to inaccurate reporting and interpretation of the data.

Regarding lifestyle habits, authors suggest that BMI and physical inactivity are important drivers of infertility [47, 131–134]. In men, obese individuals are three times more likely to show a reduction in semen quality than men of healthy weight [125]. Furthermore, moderately active men (>2 times/week) have been found to have better sperm parameters [135]. Despite this, population B (the most active population with the lowest BMI) scored only higher for the percentage of progressive motile sperm cells, equalling or even underperforming in other sperm parameters, compared to infertile males in population C. A contributing factor to mitigating the potential beneficial effects of healthy weight and exercise may be the high percentage of smokers and alcohol consumption in this population. Both habits are associated with a negative impact on sperm morphology, count, and motility [125, 135]. Once again, a more robust sample size might have provided more reliable and concordant data with the literature.

2.2 The infertile male patients

Efforts in population characterisation in the previous section showed similar lifestyle habits for populations A and C. Both showed a similar BMI, average male age over 30 (36.7 and 34.5 years, respectively), a similar auto-reported frequency in physical activity, and exposure to environmental/occupational toxic agents. Poor dietary habits were also prevalent, with similar rates of smoking and alcohol consumption. Given these two populations' relatively similar lifestyle-related factors, it is crucial to investigate and explore potential factors that could explain the significant differences observed in their sperm parameters.

From the analysis of infertile male patients' spermograms, an unexpected dip in normal semen parameters is observed for the internship period (population A), compared with the average for previous years (population C). Furthermore, population A severely and consistently underperformed in all the sperm parameters analysed. The sample size is, first and foremost, the evident and pertinent observation to make when comparing these two populations. However, significant considerations can still be derived from these data.

The uncertainty linked to a global pandemic is one likely reason for the observed disparity between populations A and C. Comparison of national birth rates in 2021 and 2022 hints at a conscious decision to postpone childbearing amongst couples [108]. Accordingly, the average age of men seeking semen analysis during the internship period was the highest ever recorded (36.7 years old). On the other hand, due to lockdown and remote working measures, one can assume that couples may have seized the opportunity to, in the *Boston Globe's* own words, 'frolic productively' [136]. This may have resulted in couples with only prolonged infertility seeking medical help, whereas those with a short duration of infertility may have conceived during this period. Boeri et al. [137] conducted a cross-sectional study and showed that the duration of infertility had a negative impact on semen parameters. In particular, a negative association was reported between sperm concentration, higher rates of azoospermia, and a longer duration of infertility. Thus, the overall observed decrease in semen quality (higher percentage of oligozoospermic and azoospermic samples) during the internship may be a direct consequence of a higher percentage of men with a longer duration of infertility seeking assistance after the COVID-19 pandemic. Unfortunately, it is impossible to verify the above assumption thoroughly, as there is no available/reliable data on the duration of infertility for couples seeking infertility treatment at CHTMAD.

Lastly, Xie et al. [138] conducted a meta-analysis study in which they reported the adverse effects of COVID-19 on semen volume, sperm concentration, sperm count, and sperm motility. And although the literature suggests that these effects may be reversible, there is not enough evidence to draw conclusive results about the long-term impacts of COVID-19 on male fertility. As such, the potential impact of SARS-CoV-2 on male patients presenting for fertility evaluation during the internship period cannot be dismissed. From this point forward, efforts should be made to record patients with past known SARS-CoV-2 infection, as some authors believe that the virus may have longer-lasting effects on the HPG axis and spermatogenesis [139, 140].

2.3 Limitations

Studies show a significant overlap in semen analysis results between fertile and infertile men [141]. As previously discussed, fertility is a couple concept, and severe alterations of the semen parameters can still be compatible with pregnancy induction (if the female partner's fertility status is optimal) and vice versa.

In addition, semen parameters are highly susceptible to intra-individual biological variability and preanalytical and analytical factors. Therefore, semen analysis must be performed in specialised laboratories following WHO guidelines and participating in quality control

programmes [129]. To add further complexity, population-based concerns have been continuously raised about possible temporal, geographic, race, or ethnic differences in semen quality [142]. Ultimately, such disparities are believed to reflect differences in environmental exposures, lifestyle, and even genetic variation [143]. As determining expected or average values for semen parameters guide physicians during the interpretation of spermograms, clear cutoff values are a necessity but very challenging to obtain [142].

Semen analysis, although the cornerstone of male fertility assessment, has critical flaws. For one, it is not a final predictive assessment of male fertility potential (unless azoospermia is detected). It is, instead, an indirect measure of male fertility that is laser-focused on the properties of semen (and sperm) and is incapable of informing on the sperm's ability to generate a healthy child. As such, vital functional features of the sperm are unaccounted for: ultrastructural defects, the ability to undergo acrosome reaction, to bind to the zona pellucida, genomic integrity, sperm aneuploidies, histone modification, protamine packaging, centriole defects, reactive oxygen species (ROS) imbalance, abnormal RNA, proteomic content and profile, methylation patterns, egg activation factors, etc. [129]. Up to 30% of men diagnosed with unexplained infertility may exhibit sperm with functional defects [144, 145].

3. DNA Damage

Lifestyle-related risk factors, such as smoking, alcohol consumption, toxic and hot environments, and others that promote increased ROS, have been linked to increasing sperm DNA damage [146]. Advanced male age, through hormonal and cellular changes brought about by the ageing process, is also a vital contributor [146].

Despite the limited sampling, donor semen samples showed a lower average rate of sperm DNA fragmentation than those provided by men seeking fertility assistance. In particular, semen donors showed a twofold lower rate of sperm DNA fragmentation. This finding aligns with several studies in which the percentage of sDF was higher in older infertile men [147]. More interestingly, Guo et al. [147] found that the sDF rate was highest in infertile men above 35, regardless of the results of semen analysis. They further concluded that men's age is more pivotal for sperm DNA integrity than routine semen parameters. In fact, despite the scarcity of data, abnormal semen samples from infertile males (population A) showed a 1.6-fold higher rate of sDF compared to abnormal semen samples from semen donors (population B). However, there is almost a 3-fold difference in the sDF rate for normozoospermic samples. Furthermore, normozoospermic and abnormal semen samples from infertile males exhibited almost identical rates of sDF (67.4% and 64.2%, respectively).

Here, despite the presence of unfavourable lifestyle factors, such as higher rates of smoking and alcohol consumption among semen donors, it is likely that the occurrence of sDF was mitigated by the younger age of the participants [148].

4. Cytogenetic and molecular studies

Male infertility may have an underlying genetic defect as a cause. Studies estimate that up to 20% of infertile men exhibit a genetic abnormality [149]. Regarding the genetic contribution to infertility, YCMD and cytogenetic abnormalities are essential aetiologies for male infertility investigations [3, 10, 150–152]. In many instances, detecting genetic alteration will not have therapeutic consequences. However, testing is performed mainly for diagnostic purposes and to assess the genetic risk to the offspring.

The frequency of chromosomal anomalies in infertile men from 2010 to 2022 for the sampled region was 2.6% (n=499) [153]. Furthermore, we found that infertile women were more prone to chromosomal abnormalities (2.9%) and that, in general, the frequency of cytogenetic abnormalities for the infertile patients was nine times the estimated incidence of chromosomal abnormalities attributed to the general (European) population (2.7 % vs 0.3%) [154].

The incidence of chromosomal anomalies in infertile patients varies considerably, country and region-wise, with studies describing rates ranging from 1.3 to 16% (**Table A.17**) [155]. Differences in sample size, ethnicity, and selection bias (most of the previous studies were conducted in specific infertile groups: couples undergoing ICSI, receiving assisted reproductive techniques, and couples with reproductive diseases) may explain the (comparatively) low frequency of chromosomal aberrations obtained for the Trás-os-Montes and Alto Douro infertile population. Markedly, this analysis was conducted in a non-selected infertile population. Furthermore, and supporting the cytogenetic findings in these infertile couples, a similar retrospective study (n=2,078) conducted in Portugal by Fernandes et al. [156] found a 2.4% incidence of chromosome abnormalities in infertile couples.

Table A.17. *Frequency of cytogenetic abnormalities in infertile couples by country.*

Study	Country	Sample characteristics	*N*	Cytogenetic anomalies (%)		
				Total	Males	Females
Peschka, et al. [157]	Germany	Couples undergoing ICSI	1,562	15.68	11.91	19.64
Clementini, et al. [86]	Italy	Couples undergoing ART	4,156	1.97	2.02	1.92
Kayed, et al. [158]	Egypt	Couples undergoing ICSI	1,218	1.18	5.21	0.91
Riccaboni, et al. [159]	Italy	Couples undergoing ART	5,420	1.37	1.5	1.3
Butnariu, et al. [160]	Romania	Couples with reproductive diseases	532	8.08	7.52	8.65
Liu et al. [155]	China	Infertile couples	29,930	3.84	6.84	0.84
Fernandes et al. [156]	Portugal	Infertile couples	2,078	2.4	?	?
Li et al. [153]	Portugal	Infertile couples	883	2.7	2.6	2.9

Regarding YCMD, they are highly linked to spermatogenetic failure, lower sperm concentration, and male factor infertility [161]. Yq microdeletions are more common in men with azoospermia than those with severe oligozoospermia, as they are rarely observed in men with a sperm count of 2.5 million sperm cells/ml. According to Punab et al. [162], 8.5% of men with fewer than 1 million sperm cells/ml have Yq microdeletions, while only 0.3% of men with a sperm count ranging from 1 to 10 million sperm cells/ml have this deletion.

Given the relatively low frequency of YCMD, it is unsurprising that no mutations were identified in the five infertile males tested during the internship period. In addition, since the introduction of AZF studies in the CHTMAD andrology laboratory in 2018, 32 men have been tested, and so far, no microdeletion has been detected. The patient selection criteria, and the small sample size are the most relevant factors responsible for the zero-detection rate of YCMD in this population. Worldwide, the reported prevalence of YCMD ranges considerably: from 0.025% to 2% in the general population of unselected men to more than 24% among infertile men [163].

Lastly, there is controversy surrounding the screening for YCMDs. Current guidelines recommend screening only in cases of severe oligozoospermia. Recent studies, however, argue

against this cut-off, as it is cost ineffective: meta-analyses have shown that there is only a significant difference in the prevalence of YCMD between males with sperm counts ranging from 0 – 1 and 1 – 5 million/ml. Therefore, the threshold for lowering to 1 million sperm/ml for YCMD testing has been continuously proposed to reduce costs without compromising sensitivity [161].

Studies estimate that nearly 50% of infertility cases may have an underlying genetic component, including chromosomal and single-gene alterations [164]. The present study revealed a low detection rate of genetic factors with karyotype analysis and YCMD screening, suggesting the presence of "hidden" genetic factors within the sampled population. In this sense, the field of genetics of male infertility has seen a shift toward the study of genetic risk factors (Single Nucleotide Polymorphisms (SNPs), Variable Number Tandem Repeats (VNTRs) and Y chromosome-linked Copy Number Variations (CNVs)), especially in idiopathic infertile males, mainly through novel approaches such as SNP array, comparative genomic hybridisation-array (array-CGH) and even next generation sequencing (NGS) [165, 166]. Understanding male reproductive health is a daunting challenge due to the intricate nature of spermatogenesis: more than 2000, both housekeeping and germ cell-specific genes are implicated [166]. Complicating matters further, these genes can be influenced by polymorphisms that directly impact male reproductive health or interact with environmental factors in complex ways [165]. As a result, research efforts to identify genetic risk factors with clinical application have yet to be successful.

5. Clinical Case

Following a miscarriage experienced by his 42-year-old female partner, a 40-year-old male health service worker underwent semen analysis, SAT, and sperm DNA damage evaluation (ACA). Furthermore, the infertile couple, facing at least two years of secondary infertility, and their product of conception underwent cytogenetic analysis.

The andrology lab records informed on the previous results of the semen analysis and the auto-reported lifestyle habits of this infertile male. The first semen analysis revealed teratozoospermia, which led to a second semen evaluation three months later, where asthenozoospermia was the main finding. The male auto-reported lifestyle habits described, for this period, no smoking or alcohol consumption and no regular physical activity. In addition, a medical history of polycystic liver disease and anticonvulsant intake (gabapentin) was also present. In the following year, a third spermiogram revealed normozoospermia. Concomitantly, the male patient reported significant differences in lifestyle habits: regular physical activity (more than two times a week), lower meat consumption (from six to only two times a week), and a cessation of anticonvulsant intake. Karyotype analysis did not detect cytogenetic alterations in the couple. The product of conception also revealed a normal karyotype: 46,XX. Extended semen analysis revealed an sDF of 69% and an aneuploidy rate of 4.5% - 7.5%. The distribution of aneuploidies for the different chromosomes analysed is shown in **Table A.7**.

Regarding the male patient's lifestyle habits, adopting healthier behaviours (lower meat consumption and physical activity) may have contributed to the observed improvement in semen quality [125, 131, 132]. Additionally, the patient discontinued the use of gabapentin. Although gabapentin can potentially affect spermatogenesis [167], studies conducted on Wistar rats have not shown any negative impact on male fertility [168]. Regrettably, no data for humans is available [167].

A growing body of evidence shows that chromatin and DNA structure defects are promising markers for male infertility regardless of the classical semen parameters (motility, concentration, and morphology) [169]. A study evaluating the predictive value of sDF tests found that ACA is the best predictor of male infertility, with a projected cut-off point of 48.5%. The value obtained for this male patient (69%) was well above the established threshold [170].

High levels of DNA fragmentation are associated with a higher incidence of chromosome anomalies in sperm from infertile men [169]. One of the main findings from this clinical case study is the nonrandom distribution of the aneuploidy rate on the analysed chromosomes. As expected, the aneuploidy rate for sex chromosomes and chromosome 21 was significantly higher (3.5 and 2.3 times higher, respectively) compared to the rates found for chromosomes 18 and 13 [79]. Despite the small number of spermatozoa scored (1/5 of the ideal), in consensus with several studies, a high score for DNA fragmentation was also found [171–173].

Pregnancy rates, both naturally and through ART, are known to be lower in couples where the male partner shows high levels of sperm DNA fragmentation [169]. Indeed, although spermatozoa with highly fragmented DNA can successfully penetrate the oocyte, embryo development is highly compromised: the oocyte shows a limited in rate and fidelity, DNA repair capacity [169, 174]. Meaning if, as studies suggest, aneuploid sperm cells display a higher percentage of fragmented DNA, even in instances of successful fertilisation, a non-viable embryo would be the result, thus preventing the development of an offspring with an abnormal genetic makeup. Therefore, we can conclude that the presence of aneuploid sperm with intact DNA is a far more harmful observation. Consequently, the frequency of this type of sperm cells is more informative of the actual risk of chromosomal abnormalities in the embryo [174].

The normal karyotype exhibited by the product of conception of this couple supports the idea that although the risk of chromosomal abnormalities is low, there is a high risk of RPL due to the high percentage of sperm with DNA damage (69%). For this couple, intracytoplasmatic sperm injection (ICSI) is highly recommended as the influence of sperm DNA damage is reduced with this technique [175]. Furthermore, high-quality oocytes (from the partner or donor) can significantly offset the negative impact of sperm DNA damage. In this case, the advanced age of the female partner bodes significant hardships even with ART. While women younger than 35 have an average success rate of 31%, women aged 41 to 44 have an average success rate of only 8% [176].

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SECTION B. MALE FERTILITY PRESERVATION

"Effects of Cryopreservation on Spermatic Parameters, DNA Integrity, and Mitochondrial Activity"

I. INTRODUCTION

Lazzaro Spallanzani, in 1776, made the first observation that human sperm motility could be preserved following a freeze/thaw cycle [177]. Since then, improvements in human sperm cryopreservation have been made. First, with the introduction of glycerol as a cryoprotectant, human spermatozoa could now be stored on dry ice (-79ºC) [178], and shortly after that, the first successful fertilisation and pregnancy were reported with the use of cryopreserved semen [179]. Since then, thousands of births worldwide have been possible using cryopreserved donor semen.

1. Cryopreservation

Long-term storage of sperm is possible only through the decrease of cellular activity by the process of freezing. To this end, liquid nitrogen sperm storage has become the standard. At -196°C there is virtually no movement of atoms or molecules [180]. The objective of every cryopreservation process, regardless of the cell type, is maintaining cellular life at subzero temperatures. To achieve this goal, it is necessary to prevent the formation of intracellular ice crystals and regulate the cell volume during freezing and thawing. Understanding these two processes and the characteristics of cells or tissues is essential to any cryopreservation protocol.

1.1 Indications for Sperm Cryopreservation

In general, a man can choose or receive services of sperm cryopreservation as a prophylactic measure before undergoing a procedure or exposure with the potential of impairing fertility (oncology treatment, surgical procedures, men in a dangerous occupation, etc.). This preventive aspect of cryopreservation, capable of ensuring the preservation and management of fertility, is crucial for long-term quality of life, particularly when very young patients are concerned [180]. Second, spermatozoa can be stored in instances of infertility treatment. For intrauterine insemination (IUI), *in vitro* fertilisation (IVF) or ICSI, sperm cryopreservation for later use may be the only viable option when dealing with cases of severe oligozoospermia, intermittent presence of motile sperm cells in semen, or partially successful treatment of infertility [3]. Exceptional circumstances of surgically collected sperm from the genital tract also benefit from cryopreservation [180].

1.2 The freeze-thaw cycle

Cells are typically suspended in a medium with solutes that lower the water freezing point to -10 to -15ºC. At these temperatures, extracellular water freezes, increasing the solute concentration (solution effect) and generating an oncotic pressure that causes the solvent to flow from inside to outside the cell. Cellular volume reduction and dehydration follow. At this moment, the cooling rate is critical: if rapid cooling of cells occurs, incomplete dehydration and intracellular ice crystal formation are the fallout; if cells are slowly cooled, however, excessive dehydration can occur and lead to permanent cellular damage (**Figure B.1**) [181, 182]. For human sperm, the optimal cooling rate is 1-10ºC/min [183].

Thawing represents another critical challenge. Water rushes back into the cell as the temperature increases, restoring intracellular volume. In this phase, intracellular ice crystal formation is also a risk. Therefore, to avoid recrystallisation injury, thawing should be fast: small ice crystals do not have enough time to recrystallise when the cell is rapidly warmed [181, 184, 185] (**Figure B.1**).

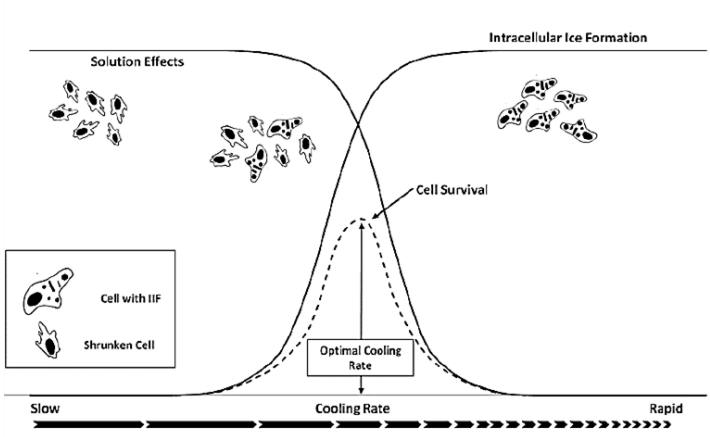

Figure B.1. Schematic representation of slow vs rapid cooling injury. Each cell type shows an optimum cooling rate due to two damaging mechanisms: solution effects and intracellular ice formation (IIF). Slow cooling damage is attributed (directly or indirectly) to elevated solute concentration. However, since slow cooling injury accumulates with increasing exposure time to damaging solute concentration, increasing the cooling rate will shorten the exposure time and increase cell viability. On the other hand, rapid cooling can lead to IIF that triggers lethal events (such as apoptosis). The optimum cooling rate balances these two competing forces: it is the rate that avoids IIF while minimising exposure to high salt concentration. Source: Hunt (2017)[185].

A delicate equilibrium between an intermediate cooling rate (fast enough to prevent extensive dehydration but slow enough to avoid intracellular ice formation) and a rapid warming rate is necessary for successful cryopreservation. In addition, morphological and physiological differences between different cell types of different species must be considered. Regarding sperm cells, different species respond differently to a freeze-thaw cycle. Thus, distinct cryopreservation protocols need to be applied [184]. That is, a good understanding of the key features of human sperm is needed to identify the challenges cryopreservation poses to these cells and to scrutinise the strategies used to shield the spermatozoa from freeze damage.

1.3 Sperm cryobiology

Human spermatozoa have a high surface area/volume ratio and high permeability to water. Thus, rapid osmotic equilibrium is achieved in the presence of cryoprotective agents (CPA), and relatively slow cooling rates (compared to cells with low surface area/volume, such as oocytes and eggs) can be safely applied [184, 186]. Furthermore, the genetic material of the sperm is highly condensed (Section A), reducing its potential for cryoinjury [181]. However, the key features responsible for granting human sperm cells an inherent resistance to cryoinjuries are (1) their low water content (50%) and (2) the presence of unsaturated fatty acids in the lipid bilayer [180] that allow sperm cells to withstand a wide range of temperature variations. However, despite all these features, at least 50% of motile sperm are damaged when subjected to cryopreservation [180].

1.4 Cryoprotective Agents (CPAs)

To protect cells and tissues from ice formation, CPAs are routinely used. Despite their varying chemical composition, all CPAs are water-soluble. Therefore, they lower the solution freezing point, displace water toward the extracellular environment, and alter the solute concentration in the liquid phase [181]. However, these agents show concentration-dependent toxicity. Two classes of CPAs are recognised:

a. Permeating CPAs: refer to low molecular weight agents (<400 g/mol) that penetrate the cell membrane. They readily cross the cell membrane, creating an osmotic gradient that promotes water movement toward the extracellular milieu, thus further lowering the freezing

point. Glycerol, dimethylsulfoxide (DMSO), ethylene glycol (EG), and 1,2 propanediol (PROH) are notorious examples [184, 186]. Regarding human sperm cryopreservation, glycerol is the most commonly employed CPA [181].

b. Non-permeating CPA: describe large molecular weight (>1000 g/mol) agents incapable of crossing the cell membrane. Their role is to increase the concentration of extracellular solutes, thus promoting cellular dehydration. This class comprises sugars such as sucrose, fructose, dextrose, trehalose, and raffinose [184, 186].

1.5 The freezing methods

The cryopreservation processes can generally be grouped into the following types:

a. Slow freezing – involves the gradual cooling of the sample over a 2-3h period, either manually or automatically through a semi-programmable freezer), from RT to -20°C, with further lowering to -80°C (at a rate of 1 to 10°C per min) before immersion in liquid nitrogen [180]. Sperm cells are exposed to less osmotic stress with slow freezing. However, ice crystals can be formed if the cooling is too steep, and cell shrinkage can occur if the cooling is too slow [184].

b. Rapid freezing – this technique strives to minimise the toxicity caused by the cryoprotectant and mitigate osmotic membrane damage. After adding, drop by drop, the cryoprotectant, the sample is brought in direct contact with nitrogen vapours (-80°C) for 30 min before immersion in liquid nitrogen [181].

c. Vitrification – allows the sperm to be cooled at a fast rate (-1000°C/min) to achieve a glass-like solidification without the formation of ice crystals. Thus, small sample volumes are dropped directly into liquid nitrogen to achieve such high-rate cooling [180].

2. Sperm Cryoinjury

Sperm damage due to cryopreservation results from a combination of four factors: osmotic stress, intracellular and extracellular ice formation, cryoprotectant toxicity, and oxidative stress [187–189]. Following a freeze and thaw cycle, motility becomes the most affected parameter [190, 191]. The decline is even more significant in patients with poor sperm quality pre-freeze. This overall decline in motility is attributed mainly to mitochondrial damage [192]. In addition, elevated ROS levels are a common observation in cryopreserved sperm and are believed to contribute to (sub)lethal cellular damage [191, 193–195]. Osmotic and oxidative stress are the main culprits in excessive ROS production [188, 196–198].

2.1 Morphology damage

Morphology evaluations consistently report an increase in sperm with midpiece detachment and coiled tails. In addition, transmission electron microscopy and scanning electron microscopy studies point to various ultrastructural abnormalities after thawing: plasma membrane decomposition, anomalies in chromatin condensation, anomalies in the acrosomal membrane and content, subacrosomal swelling, deformations in neck, tail, and mitochondrion damage [199].

2.2 CPA toxicity

Prior to cryopreservation, the highest concentration of a CPA that a sperm cell will tolerate is limited, and during freezing, this concentration will increase as ice forms. The maximum concentration that can be achieved without compromising the cell viability depends on the

temperature (chemical toxicity is reduced at low temperatures) and rate of CPA addition and removal (osmotic shock results in cell damage) [200, 201].

2.3 Mitochondrial damage

Mitochondria create the energy necessary for sperm movement. Therefore, any impairment to the mitochondrion metabolism leads to decreased metabolism. However, these organelles are simultaneously involved in the processes of apoptosis and the production of free radicals. Two types of damage occur in sperm mitochondria after cryopreservation: direct damage to the inner and outer membranes of the mitochondrion and mitochondrial DNA; and indirect damage through loss of genetic coding for mitochondrial activity [184]. Mitochondrial and plasma membranes share a similar sensitivity to cryopreservation: the membrane becomes less fluid under cold conditions. This results in changes in membrane potential and the release of free oxygen radicals [180, 186], leading to damage to the plasma membrane and disruption of the axonemal structure [202].

2.4 ROS damage

Cell cooling causes physical damage and produces excessive free radicals that disrupt vital chemical structures in the sperm cell. In particular, they affect membrane lipids, proteins, and nucleic acids [184]. Under normal physiological conditions, the formation and disintegration of free radicals remain in equilibrium. However, the cryopreservation process displaces this equilibrium toward the continuous formation and association of free oxygen radicals (natural antioxidant defence cell mechanisms are overrun) and the deterioration of cell structures essentially through lipid peroxidation [203]. Furthermore, it has been reported that due to disturbances in the selective-membrane permeability of the spermatozoa, cryopreserved sperm cells cannot exert normal capacitation and fertilisation processes. A high calcium concentration in the cytosolic environment results in premature acrosome reaction and sperm hyperactivation [204]. All these events have moved the spotlight towards using calcium channel blockers and antioxidants in cryopreservation protocols [184].

2.5 DNA damage

Regarding sperm DNA damage, there is a marked lack of consensus. Several authors believe sperm DNA damage increases after a cycle of freeze/thaw [188, 196, 205]. This decrease in DNA quality is mediated mainly through oxidative stress rather than apoptosis [188]. Cryopreservation leads to the production of free oxygen radicals that damage sperm DNA [206, 207]. And in the presence of a weak DNA repair mechanism, sperm cells are vulnerable to oxidative attacks [181, 184]. For others, damage in sperm DNA is only observed in infertile men: as pointed out by Kalthur et al. [187], morphologically abnormal sperm are more prone to DNA damage as compared to sperm with normal morphology. In contrast, a third group of research defends that a freeze-thaw cycle does not harm the DNA integrity of sperm cells [208].

3. The Role of Antioxidants in Sperm Cryopreservation

Antioxidants exert their effect by preventing free oxygen radicals from starting chain reactions, stopping chain reactions that have already begun, and breaking down and reducing local oxygen concentration [209].

The cell's antioxidant defence systems are complex and can be enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic. In sperm (and semen plasma), the enzyme system consists of glutathione peroxidase, superoxide dismutase, and catalase. They convert oxidised metabolic products to water with the assistance of cofactors (iron, zinc, copper, and manganese) [210]. On the other hand, non-enzymatic antioxidants

describe substances (endogenous or exogenous) that intercept and terminate free radical chain reactions (vitamin A, E, C, flavonoids, carotenoids, glutathione, curcumin, melatonin, bilirubin, plant polyphenols, uric acid, theaflavin, among many others) [209, 211].

Sperm naturally produce ROS in minimal amounts, as they are essential for sperm capacitation initiation, motility, regulation of sperm maturation, enhancement of cell signalling pathways, apoptosis, and sperm chromatin condensation [212]. In contrast, these germ cells contain low levels of enzymatic antioxidants, making them highly vulnerable to oxidative attacks. Sperm DNA is especially susceptible to oxidative damage because the highly condensed nuclear structure of the sperm prohibits the enzymatic repair of damaged DNA [213]. Thus, to maintain functionality, sperm cells need to balance their redox potential [212]. Maintaining this delicate balance becomes particularly crucial when these cells are exposed to a cycle of freezing and thawing. Furthermore, processing semen before cryopreservation removes the seminal plasma that, through its antioxidant properties, shields sperm cells against ROS [214]. The addition of antioxidants to freezing mediums has become common practice to boost sperm antioxidant defence mechanisms and preserve, to a satisfactory degree, the biological potential of sperm cells [193, 215–225]. Plant-derived and synthetic compounds are regularly used in sperm cryopreservation protocols of various species as valuable sources of antioxidants [226].

Here, two non-enzymatic antioxidants, Vitamin E (VE) and Astaxanthin (ASTX) (**Figure B.2**), were used, and their effect on sperm motility, concentration, vitality, morphology, and DNA integrity was evaluated.

3.1 Vitamin E (VE)

VE refers to a collective group of fat-soluble compounds with different antioxidant activities. VE is found mainly in nuts, seeds, and vegetable oils. Eight naturally occurring forms of VE are known: the alpha, beta, gamma, and delta classes of tocopherol (saturated side chain) and tocotrienol (unsaturated side chain) [227]. In humans, there is a preferential distribution of alpha-tocopherol forms due to the faster metabolism of the other forms and the presence of specific transfer proteins for alpha-tocopherol [227]. VE is a potent chain-breaking antioxidant capable of hindering ROS production and propagation of free radical reactions [228]. In particular, alpha-tocopherol inhibits the production of new free radicals, whereas the gamma forms are responsible for neutralising existing free radicals. Located primarily in cell organelle membranes, alpha-tocopherol protects these structures from lipid peroxidation through its peroxyl radical-scavenging activity [228]. Thus, VE is routinely used to counteract numerous conditions/diseases associated with ROS attacks [227].

3.2 Astaxanthin (ASTX)

ASTX is a marine xanthophyll (oxygenated carotenoid) produced by algal species (*Haematococcus pluvialis*, *Chlorella zofingiensis*, and *Chlorococcum*), bacteria, and fungi that confers, through the food chain, the rich pink colour to several (primarily aquatic) species (from salmonids to flamingos) [229]. Its unique molecular structure allows it to span over the cell's lipid bilayer membrane and effectively shield against oxidative attacks [229, 230] (**Figure B.2. Location of Vitamin E and Astaxanthin in cell membranes.** Adapted from Budriesi et al. [229].). Unlike most antioxidants, it can scavenge and quench ROS in both the inner and outer layers of the cellular membrane [231]. The antioxidant has received increasing attention as an effective molecule to counteract and dilute the effects of oxidative stress-related conditions [231]. Numerous studies have been conducted on its anticancer, antidiabetic, anti-inflammatory, immune-stimulating, and antioxidant properties, most suggesting a potential therapeutic effect [232–234].

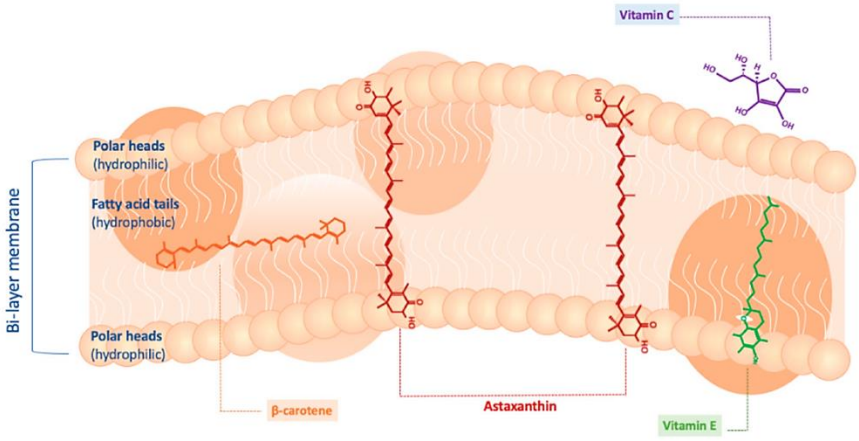

Figure B.2. Location of Vitamin E and Astaxanthin in cell membranes. *Adapted from Budriesi et al.* [229].

II. OBJECTIVES

Cryopreservation is a complex procedure that requires the precise regulation of many factors to ensure (minimal) success. This section aims to elucidate the challenges that male fertility preservation currently faces and assess the impact of ASTX, DMSO and VE supplementation on sperm post-thaw parameters.

In short, the following goals were outlined: (1) review fertility preservation processes, detail instances where they are employed and their impact on sperm parameters, (2) describe current protocols on sperm cryopreservation and strategies in use to protect sperm cells from freeze damage, (3) assess the impact of cryopreservation on sperm quality parameters such as motility, vitality, morphology, and DNA integrity, (4) study the impact of DMSO, ASTX, and VE on sperm cryopreservation (offer detailed analysis on the main findings of the study and comment on its main limitations and drawbacks), and (5) comment on the future of male fertility preservation techniques and protocols.

III. MATERIAL & METHODS

1. Study Participants

The study included normozoospermic semen samples from men who sought fertility evaluation at CHTMAD during the internship period and semen samples from normozoospermic semen donors. To eliminate factors that could affect ROS generation, men with a history of drug addiction, smoking, alcohol consumption, prolonged diseases, drug consumption (including vitamins), and any sample with leukocytospermia were excluded. Furthermore, samples that did not liquefy after 30-40 min of incubation at 37°C, with a volume below 1.5 ml and or sperm concentration less than 20 million sperm cells/ml, were also removed from the study. Consequently, from an initial pool of 18 normozoospermic semen samples, only six were enrolled in the study.

2. The Antioxidants

Two antioxidants, VE and ASTX, were used to explore their potential protective effect when used alone or combined during the cryopreservation of human sperm cells.

2.1 Vitamin E

Here, sperm freezing medium (SFM) (ORIGIO, Måløv, Denmark) was supplemented with 5 mM VE (Sigma-Aldrich Company, St. Louis, MO; Cat. No.T-3251). Two stock solutions of VE in SFM (one for mixture with ASTX and one for VE alone) were prepared weekly and kept at 4°C. When necessary, 220 µL of VE-supplemented SFM was left at RT for at least two hours before use.

2.2 Astaxanthin

For the proposed study, the SFM (ORIGIO, Måløv, Denmark) was supplemented with 0, 15 and 25 µM of ASTX (Sigma-Aldrich Company, St. Louis, MO; Cat. No. SML0982-50MG) solubilised in 2.5 and 5.0% DMSO. For this, stock solutions were prepared and then frozen in aliquots of 30 µL each. When necessary, the aliquots were pre-warmed in a 37°C water bath for 5 min, then added to SFM and left at RT for at least two hours. The experimental groups with 0 µM ASTX had the SFM supplemented with 2.5 and 5.0% (v/v) DMSO, respectively.

3. Experimental Design

The experiment was divided into two sections. The first section describes efforts to optimise the cryopreservation process. The last one illustrates the developed experimental work (**Table. B.1**).

3.1 Section 1: Optimisation of the Cryopreservation Protocol

Three semen samples were subjected to swim-up and different wash regimes to determine the least harmful pre-freezing procedure. Appendix J. Direct Swim-up and Simple Wash Protocol contains detailed protocol. In addition, a short, straightforward literature review was conducted to identify the most commonly applied protocol and techniques in human sperm cryopreservation studies.

3.2 Section 2: Study Design

Three semen samples had their freezing medium supplemented with different concentrations of astaxanthin (0, 15, 25 µM) solubilised in 2.5% (v/v) DMSO; and a mixture of both antioxidants (25 µM ASTX at 2.5% DMSO + 5 mM VE – group D). Similarly, for the 5.0% DMSO

experimental groups, three semen samples had their freezing medium supplemented with different concentrations of astaxanthin (0, 15, 25 μM) solubilised in 5.0% (v/v) DMSO (groups E-G and a mixture of both antioxidants (25 μM ASTX at 5.0% DMSO + 5 mM VE – group H). Lastly, a control group (n=3) with only SFM and a VE group (n=6) with 5 mM VE added to SFM was created (**Table. B.1**).

Table. B.1. *Experimental Design.*

Groups	**Control**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**VE**
	SFM (1:1)									
DMSO (%)	-	2.5% DMSO				5.0% DMSO				-
ASTX (μM)	-	0	15	25	25	0	15	25	25	-
VE (mM)	-	-	-	-	5	-	-	-	5	5
Samples	1,2,5	1 – 3				4 – 6				1 – 6

Note: The experimental groups created in the present study underwent cryopreservation in the presence of SFM. Control group (n=3) was cryopreserved with only SFM. Groups A-D (n=3) were cryopreserved with SFM supplemented with 2.5% DMSO and varying concentrations of antioxidants, while groups E-H (n=3) were cryopreserved with SFM supplemented with 5.0% DMSO and different concentrations of antioxidants. In the VE group (n=6), SFM was supplemented with 5 mM VE.

4. Cryopreservation Protocol

The performed cryopreservation consisted of three main stages: pre-freezing procedures, rapid freezing protocol, and thawing procedures. Each phase involved careful planning and the adoption of specific measures to minimise sperm injury. All materials (centrifuge tubes, glass slides, etc.) and reagents used were prewarmed, and all the medium was slowly added to the semen sample (in some instances, drop by drop) to allow for gradual osmotic adjustment.

4.1 Pre-Freezing

Before the reception of the semen sample, supplemented and non-supplemented SFM was pre-warmed for a minimum of 2h at RT. Following the reception and liquefaction of the sample, semen analysis was carried out according to WHO guidelines (Section A). Motility, viability, concentration, and morphology were recorded. Subsequently, two washes were performed, both at 300 g (the first for 8 min and the last for 5 min) using a Sperm Preparation Medium (SPM) (ORIGIO, Måløv, Denmark). An aliquot of 100 μL was first reserved for ACA procedures, and then SPM was added to adjust the sperm concentration to 20 million sperm cells/ml. The processed semen sample was hereafter kept at RT (for 10 min), and sperm motility, vitality, and concentration were again checked. These were considered the pre-freezing parameters.

4.2 Rapid Freeze Protocol

The processed semen sample was divided into five (or six, for the three samples where the original semen volume and sperm concentration allowed the creation of a control group – **Table. B.1**) aliquots. Supplemented (and, when applicable, non-supplemented) SFM (ORIGIO, Måløv, Denmark) – HEPES buffered medium with glycerol, sucrose, and human serum albumin – was added 1:1 (v/v), drop by drop, to processed semen aliquots, and the solution was carefully mixed after each addition. The mixture was left at RT for a minimum of 10 min and then loaded into cryotubes. The cryotubes were then suspended horizontally above the surface of liquid nitrogen for 30 min and finally transferred to liquid nitrogen and stored at -196°C for two weeks.

4.3 Thawing

The cryotubes were warmed in a water bath (37°C) for 5 min. The thawed semen was removed and washed in pre-warmed SPM, 300 g, for 5 min. Next, the pellet was gently and gradually resuspended in 200 μL of SPM and semen analysis was performed. For each aliquot, 50 μL was reserved for ACA.

5. Statistical Analysis

The normality of the data was analysed by the Shapiro-Wilk test. Parametric data were analysed by one-way ANOVA test and Tukey post-test. In contrast, the nonparametric data were analysed using the Kruskal-Wallis test to evaluate the significant differences between groups. The term 'statistically significant' was used to signify a p-value <0.05. All analyses were performed using the R statistical software (version 4.2.2; R Foundation for Statistical Computing, Vienna, Austria).

IV. RESULTS

1. Sperm cryopreservation protocol

1.1 Literature review on human sperm cryopreservation protocols

Human sperm cryopreservation protocols vary significantly. The literature review of human cryopreservation medium supplementation studies identified important trends.

A total of 36 studies, from 1994 to 2021, were analysed [188, 194, 205, 220, 235–266] (**Table. B.2**) Vitamin E was the antioxidant most studied (n=3), followed by glutathione, resveratrol, ascorbic acid, melatonin, mito-TEMPO, and myoinositol (n=2, each). Regarding the cryopreservation protocol, the rapid freezing method (79%), medium supplementation before cryopreservation (87%), and thawing after two weeks (29%) were the preferred strategy among researchers (**Figure B.3**). It should be noted that the vague term "extended storage" (without a specification of freezing time) was found to be a common phenomenon amongst the reviewed studies (32%).

Table. B.2. *List of additives commonly employed in sperm cryopreservation studies.*

Additives		
Alpha lipoic acid	Green tea extract	Potent Humanin analogue
Ascorbic acid	*Holotheria parva* coelomic cavity extract	Reduced glutathione
Brain-derived neurotrophic factor (BDNF)	Hypotaurine	Resveratrol
Butylated Hydroxytoluene	Isoflavone genistein	Sericin
Caffeine	L-carnitine	Stromal cell-derived factor-1α
Canthaxanthin	Leptin	TAT-Peroxiredoxin 2 fusion protein
Catalase	Melatonin	Tempol
Chlorogenic acid	Mito-TEMPO	Vitamin B12
Curcumin	Myoinositol	Vitamin E
Genistein	Nerve growth factor	Zinc

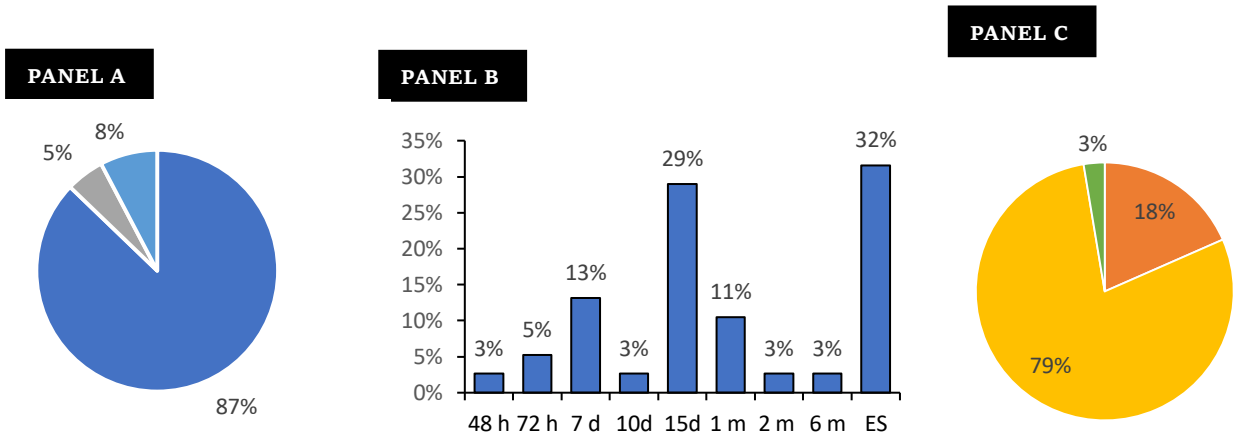

Figure B.3. Strategies employed in sperm cryopreservation studies. *Panel A – Order of additive supplementation. Blue – before freezing; grey – before and after freezing; light blue – after freezing. Panel B – Duration of cryopreservation. h – hours; d – days; m – months; ES – extended storage. Panel C – Methods of cryopreservation. Yellow – rapid freezing; green– vitrification; orange – slow freezing.*

1.2 Washing procedures

Three normozoospermic semen samples were used to evaluate the impact of swim-up and different wash regimes on motility and morphology.

1.2.1 Swim-up

Comparison between fresh semen and semen processed using the swim-up method revealed differences in motility parameters (**Figure B.4**). The swim-up technique resulted in a higher percentage of progressive (86% vs 76%) and total motility (95% vs 84%) than fresh semen (p-values of 0.017 and 0.014, respectively). When examining specific motility categories, it was observed that the swim-up method reduced immotile sperm (16% vs 5%), while non-progressive sperm share remained the same (8%).

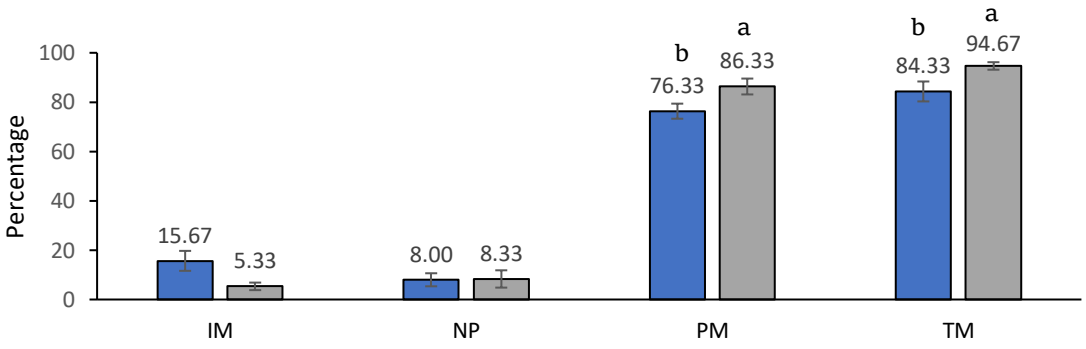

Figure B.4. Swim-up effect on sperm motility. Blue – fresh semen samples, Grey – semen samples after swim-up. Bars represent standard deviation. Different letters indicate statistically significant differences (p<0.05). IM – immotile; NP – Non-progressive; PM – Progressive motility; TM – Total Motility

Regarding morphology, the swim-up method showed a higher percentage of sperm with typical morphology compared to fresh semen (14% vs 5%) (p-value = 0.0362) (**Figure B.5**). Furthermore, a lower percentage of sperm with head anomalies was observed (80% vs 93%) (p-value=0.0354) and a slight increase in the percentage of sperm with tail anomalies (9% vs 4%) (p-value = 0.0344). The swim-up method showed no significant changes in the percentage of sperm with midpiece abnormalities or ERC anomalies compared to fresh semen.

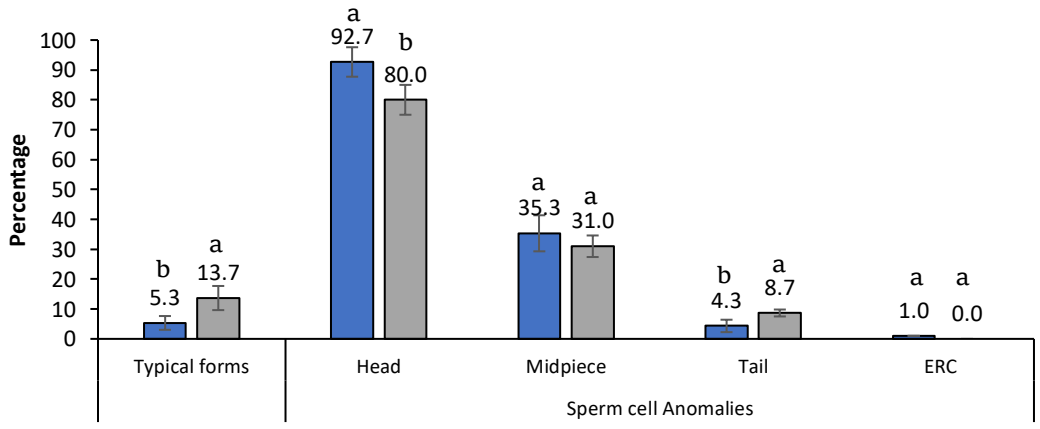

Figure B.5. Swim-up effect on sperm morphology. *Blue – fresh semen samples; grey – semen samples after swim-up. ERC – Excess Residual Cytoplasm. Bars represent standard deviation. Different letters indicate statistically significant differences (p<0.05).*

1.2.2 Wash regimes

The sperm total motility was evaluated after two washes under different centrifugation conditions: (1) both washes at 300 g for 5 minutes, (2) 300 g wash for 8 minutes followed by an additional 5-minute wash and two washes at 300 g for 8 minutes. As the duration of centrifugation increased, a decrease in total motility was observed (**Figure B.6**). Centrifugation at 300 g for 5 minutes resulted in total motility of 73%, while the centrifugation at 300 g for 8 minutes, followed by an additional 5-minute wash showed a further decrease to 67%. The most significant reduction in total motility was observed with centrifugation at 300 g for 8 minutes, resulting in total motility of 52%.

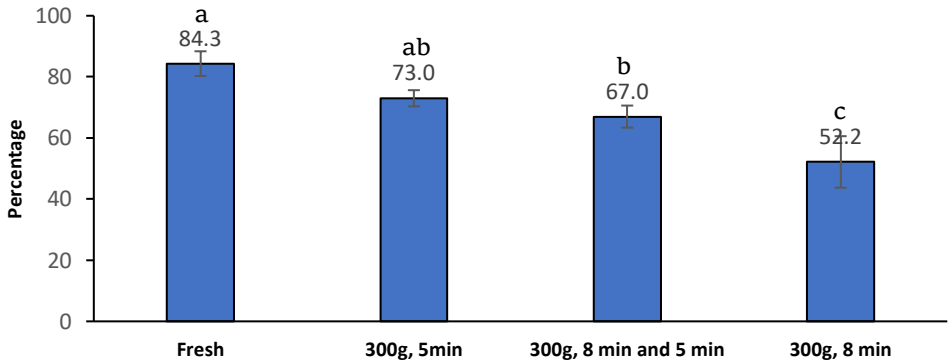

Figure B.6. Effect of different wash regimes on sperm total motility. *Two washes were performed. Different letters indicate statistically significant differences (p<0.05).*

2. Cryopreservation

2.1 Participants' semen profile

Six normozoospermic semen samples were enrolled in the study. The mean age and body mass index (BMI) of the subjects were calculated as 32.6 (between 18 and 46) years and 25.4 (between 23 and 30) kg/m^2, respectively. Semen parameters (mean, range) were evaluated for sperm volume (ml), sperm count, sperm total motility, and sDF (**Table. B.3.**). On average, the semen samples enrolled in our study exhibited a total motility of 75.5%, a vitality of 82.3%, a sperm count of 46 million sperm cells/ml, and 4.7% of normal forms.

Table. B.3. *Study participants' semen parameters.*

Volume (mL)	Total motility (%)	Vitality	Sperm Count (x10^6/mL)	% Normal forms
3.0 (1.5 – 4.6)	75.5 (64 – 84)	82.3 (67.1 – 89.0)	45.9 (23.0 – 61.5)	4.7 (4 – 7)

2.2 Pre-freeze and post-thaw semen parameters

The analysis of pre-freeze and post-thaw semen parameters showed significant changes in various sperm characteristics (**Table. B.4**). The total motility, vitality, and concentration of sperm decreased significantly after thawing. The percentage of total motility decreased by 78%, while vitality and concentration decreased by 76% each. Regarding sperm morphology, the percentage of normal forms decreased by 43% after thawing (from 6.0 to 3.4). However, specific anomalies exhibited different patterns. The head anomaly increased slightly by 5%, while the midpiece anomaly decreased by 12%. Tail anomalies increased by 40%, and ERC decreased by 41%. The analysis of sDF (ACA) revealed a significant increase of 140% in post-thaw samples compared to pre-freeze samples.

Table. B.4. *Pre-freeze and post-thaw semen parameters and respective variation.*

	Pre-Freeze (*n*=6)	**Post-Thaw (*n*=3)**	**Variation (%)**
Total motility (%)	64.46 ± 11.78	14.03 ± 2.89	-78
Vitality (%)	71.58 ± 8.55	17.17 ± 4.25	-76
Concentration (10^6/mL)	20.35 ± 3.26	4.95 ± 0.86	-76
Morphology (%)			
Normal Forms	6.0 ± 3.0	3.4 ± 1.9	-43
Anomalies			
Head	88.9 ± 4.9	93.4 ± 4.0	+5
Midpiece	53.5 ± 11.2	46.9 ± 14.7	-12
Tail	15.2 ± 6.0	21.3 ± 6.8	+40
ERC	0.7 ± 1.0	0.4 ± 0.7	-41
sDF (%)	25.5 ± 11.04	60.8 ± 7.0	+140

ERC, Excess Residual Cytoplasm; sDF, sperm DNA fragmentation.

2.3 DMSO Effect

Table. B.5 compares semen parameters between the control group and two different concentrations of DMSO: 2.5% DMSO (A) and 5.0% DMSO (E). Regarding total motility, the control group showed lower values (14.03 ± 2.89%) than the 5.0% DMSO group (20.8 ± 6.1%) but similar to the 2.5% DMSO group (11.2 ± 3.6%). For vitality, the control group scored 17.17 ± 4.25%, while the 2.5% DMSO group exhibited a slightly higher value (19.0 ± 0.5%). In contrast, the 5.0% DMSO group showed the highest vitality (40.8 ± 3.9%). Additionally, the control group demonstrated a concentration of 4.95 ± 0.9 million/ml, slightly higher than the 2,5% DMSO group (4.3 ± 1.5 million/ml) but lower than the 5.0% DMSO group (6.68 ± 5.1 million/ml). The percentages of normal forms, head anomalies, midpiece anomalies, tail anomalies, and ERC anomalies varied between the three groups, with the highest percentage of tail anomalies found in the 5.0% DMSO group (37.7%). Lastly, for the assessment of genomic integrity, DMSO 5.0% showed the lowest percentage of sperm DNA fragmentation (44.5%).

2.4 Antioxidant Effect

Regarding the different antioxidant treatments, the F-H groups showed a protective effect against cryopreservation (**Table. B.6**). In contrast, VE showed no significant improvement over the control values. The B-D groups showed only a mild reduction in the sDF rate.

Regarding total motility, groups F (17.2 ± 10.4%) and G (18.7 ± 5.5%) exhibited higher percentages than the other treatments. For vitality, groups F (38.7 ± 7.1%), G (37.7 ± 3.7%), and H (36.9 ± 3.5%) scored the highest. In terms of concentration, group F (7.7 ± 4.0 million sperm cells/mL) showed the highest value among all treatments, followed by G (6.36 ± 2.0 million sperm cells/mL) and H (6.0 ± 4.8 million sperm cells/mL) groups.

Concerning morphology, only the F-H groups exhibited, on average, a percentage of typical sperm greater than 4% (5.3, 4.7, and 5.0%, respectively). In contrast, this was also the group with the highest percentage of tail abnormalities (34.7, 42.3, and 43.3%, respectively). Finally, sperm DNA damage was the highest in the control group (60.8%). In contrast, the F-H groups exhibited the lowest percentage of sDF after a freeze-thaw cycle (29.8, 34.3, and 41.3%, respectively).

Table. B.5. *DMSO effect on post-thaw semen parameters*

		2.5% DMSO	**5.0% DMSO**
	Control	A	E
Total motility (%)	14.03 ± 2.89	11.2 ± 3.6	20.8 ± 6.1
Vitality (%)	17.17 ± 4.25^{b}	19.0 ± 0.5^{ab}	40.8 ± 3.9^{a}
Concentration (x10^{6}/mL)	4.95 ± 0.9	4.3 ± 1.5	6.68 ± 5.1
Morphology (%)			
Typical Forms	3.4 ± 1.9	3.8 ± 2.3	5.3 ± 1.5
Anomalies			
Head	93.4 ± 4.0	92.2 ± 4.3	88.7 ± 2.1
Midpiece	46.9 ± 14.7	41.7 ± 15.2	34.3 ± 10.0
Tail	21.3 ± 6.8	27.2 ± 9.4	37.7 ± 7.2
ERC	0.4 ± 0.7	0.2 ± 0.3	0.0 ± 0.0
sDF (%)	60.8 ± 7.0^{a}	50.6 ± 6.9^{ab}	44.5 ± 12.5^{b}

Different letters indicate statistically significant differences (p<0.05). ERC, Excess Residual Cytoplasm; sDF, sperm DNA fragmentation.

Table. B.6. *Antioxidant effect on post-thaw semen parameters*

		2.5% DMSO			**5.0% DMSO**			
Groups	Control	B	C	D	F	G	H	VE
TM (%)	14.0±2.9	10.8±0.9	13.1±9.1	11.6±3.1	17.2±10.4	18.7±5.5	11.7±2.2	10.0±3.9
Vitality (%)	17.2±4.3	18.3±5.4	21.7±10.9	16.8±4.9	38.7±7.1	37.7±3.7	36.9±3.5	14.0±5.3
Conc.(x10^{6}/ml)	4.95 ± 0.9	4.7 ± 1.5	3.4 ±1.3	3.8 ± 0.8	7.7±4.0	6.36±2.0	6.0±4.8	4.7±3.4
Morphology (%)								
Normal Forms	3.4±1.9	4.3±2.4	2.5±0.7	2.3±0.5	5.3±3.2	4.7±0.6	5.0±2.0	3.7±1.2
Anomalies								
Head	93.4 ±4.0	93.4 ±4.7	96.2 ±1.8	96.5 ±0.9	88.0±6.1	88.3±1.5	89.3±4.5	92.1±3.5
Midpiece	46.9 ±14.7	29.1 ±11.8	37.6 ±10.1	38.4 ±21.6	44.3±15.9	40.3±6.0	37.7±6.1	34.3±7.1
Tail	21.3 ±6.8	23.8 ±4.4	21.6 ±6.1	31.9 ±11.6	34.7±8.4	42.3±8.1	43.3±9.0	32.0±8.6
ERC	0.2 ±0.7	0.8 ±0.7	0.5 ±1.4	1.2 ±1.3	0.0±0.0	0.7±0.6	0.0±0.0	0.2±0.4
sDF (%)	60.8±7.0^{a}	48.7±9.4^{ab}	50.1±7.6^{ab}	51.9±5.4^{ab}	29.8±18.1^{cb}	34.3±17.6^{b}	41.3 ±7.3^{ab}	50.4±10.4^{ab}

Different letters indicate statistically significant differences (p<0.05). TM, total motility; VE, vitamin E; ERC, Excess Residual Cytoplasm; Conc., concentration; sDF, sperm DNA fragmentation

V. DISCUSSION

The determination of the optimal cryopreservation protocol is a challenging process. Numerous CPAs, additives, and freezing and thawing methods are described for sperm cryopreservation. To make matters worse, the WHO guidelines offer only instructions for freezing methods and suggest using any commercially available CPA [62]. Therefore, a comprehensive analysis of the methodologies used in several publications was performed to aid the experimental design of the proposed study on freezing medium supplementation. Factors such as cost-effectiveness, equipment requirements, availability of reagents, and time management were carefully considered during this evaluation process.

1. Human sperm cryopreservation protocols & optimisation

Based on the analysis of available evidence and published studies, the rapid freezing protocol emerged as a promising method due to its simple freezing process, reduced operation time, and proven cost-effective approach [267]. Furthermore, following the trend observed in most published studies, we supplemented the freezing medium before the freeze-thaw cycle.

Before cryopreservation, sperm samples undergo a process of washing and processing to eliminate round cells, leukocytes, dead cells, debris, and seminal plasma to obtain good-quality sperm pre-freezing. Mainly because abnormal sperm and leukocytes produce ROS that cause sperm damage and DNA fragmentation [268]. Similarly, the washing and preparation of post-thawed spermatozoa are also vital. In this instance, sperm preparation is used to discard the cryoprotectant (at higher temperatures, CPAs display sperm toxicity) and to select the best quality sperm (important for ART) [180].

Swim-up is the preferred method of sperm selection [268–270]. In this technique, sperm are introduced into an overlaid medium and allowed to swim upward. Then, this upper fraction becomes primarily comprised of sperm cells with improved motility, a higher percentage of normal morphology and improved *in vitro* fertilisation rates [271]. The obtained results from the swim-up technique performed on three normozoospermic semen samples corroborate this observation (motility and morphology wise). However, due to concerns regarding its low yield (it requires high sperm count and motility) and the availability of reagents, this sperm purification method was eventually discontinued, despite its initial successful implementation.

The simple wash method is easier to perform and yields higher sperm concentrations with less preparation time. Two washes are generally recommended for the complete removal of seminal plasma [268]. However, centrifugation has been shown to generate ROS, and studies dating back to 1993 advocate for a shorter centrifugation period in sperm preparation procedures [272]. With this in mind, three wash regimens were tested. Chaparro & Kim [273] tested different centrifugation regimes and found that prolonged centrifugation decreases sperm motility. Such a finding resembles those of our study. The highest decrease in sperm total motility was observed after two 8-minute washes (from 84.3%, in fresh samples, to 52.2%), while the most negligible variation in motility was observed after 5-minute washes (from 84.3% to 73.0%). However, the chosen time regimen for sperm washing was an 8-minute wash followed by another 5-minute wash. Unfortunately, incomplete pelleting occurs when performing two washes of only 5 minutes each, severely compromising sperm yield. Therefore, consistently cryopreserving 20 million sperm cells/ml for each experimental group would pose a significant challenge.

Lastly, in line with the beneficial effects reported in numerous studies, VE was selected primarily not only to evaluate the quality of the cryopreservation protocol used but to assess the reproducibility of previous findings.

2. Cryopreservation Effects

Sperm cryopreservation is a challenging process. Freezing injury is frequent and impairs sperm function. Here, a freezing and thawing cycle caused a significant decrease in semen quality (**Table. B.4**).

2.1 Motility

Depending on the initial semen quality and the freezing/thawing protocols used, sperm cryopreservation causes a decrease in motile sperm from 30 to 50% [274]. However, for the analysed samples, this decrease was significantly higher. Centrifugation is known to cause significant stress on sperm by weakening the plasma membrane and increasing lipid peroxidation through ROS production [275]. For this study, because the possible protective effect of seminal plasma had to be eliminated to assess the impact of antioxidant freezing medium supplementation correctly, sperm cells were subjected to 3 washes (2 consecutive pre-freeze washes and one post-thaw wash). Consequently, centrifugation likely introduced sublethal damage further exacerbated during cryopreservation [272]. For this reason, methods that avoid centrifugation (swim-up) are usually preferred [272].

2.2 Concentration

The conscious effort to pursue cryopreservation with an equal number of sperm cells in an equal volume of semen was due to reports of the impact of different sperm concentrations on post-thaw viability and motility of cryopreserved sperm [276]. Post-thaw variation in concentration is vital because, in theory, it is a parameter capable of informing on the presence of loose heads. However, the force and duration of centrifugation greatly determine sperm recovery and yield. Lower centrifugal forces and a short centrifugation time prevent complete pelleting; therefore, sperm loss occurs upon supernatant removal. On the other hand, the opposite harms sperm motility and vitality. As such, and because the post-thaw wash was performed with the lowest force for the shortest time (300 g, 5 min), it is unlikely that complete pelleting of the sample occurred. Thus, the variation in sperm concentration provides no relevant and reliable information [277].

2.3 Vitality

Henry et al. reported, in 1993 [183], that cryopreservation similarly affects motility, membrane integrity, and mitochondrial function. Consistent with these findings, post-thaw damage to sperm vitality was identical to the reduction in sperm motility. Induction of an apoptotic pathway and lipid peroxidation through excessive ROS production is the most agreed-upon mechanism responsible for the drop in sperm vitality [274]. Furthermore, because mitochondria are known to initiate cell death by apoptosis [278], the obtained results mirror this biological fact. The low percentage of alive but immotile spermatozoa speaks of the interdependence between the integrity of the plasma membrane and functional mitochondria. Cryoinjury to mitochondria is believed to trigger an apoptosis-like mechanism into motion, causing, post-thaw, further loss of mitochondrial function and damage to plasma membranes. As a result, the decrease in motility is, as observed, concomitantly followed by a similar decrease in vitality [192].

2.4 Morphology

Regarding sperm morphology, the coiling up of the tail is the most common observed abnormality, followed by loose heads. This tail abnormality is mainly due to osmotic changes.

Consequently, the HOS test in cryopreserved semen samples is highly prone to false positives and was therefore performed only in fresh samples. Furthermore, the observed detachment of the head and tail after thawing is probably attributed to ice crystal formation during freezing.

2.5 Genomic Integrity

DNA damage was the parameter with the highest post-thaw increase. The negative impact of cryopreservation on genomic integrity is well documented and is consistent with the presented results [188, 190, 199].

3. DMSO Impact

The detailed findings reveal, for our study, an unexpected influence of DMSO concentration on sperm post-thaw parameters (**Table. B.5**). Five per cent DMSO was necessary to improve sperm vitality and morphology and significantly decrease DNA damage. Furthermore, the experimental group with 5.0% DMSO showed the highest variation between the observed values for motility and vitality. On average, vitality scored 21% higher than motility. This discrepancy was not present in the remaining groups (average of 3.1% for control, 7.3% for 2.5% DMSO groups and 6.5% for the VE group) and is indicative of a high percentage of alive but immotile sperm in the E-H groups (**Figure B.7**). As stated above, due to the dominant role of mitochondria in the initiation of apoptosis, damage to this organelle increases the likelihood of cellular death, and, consequently, decreases sperm vitality. However, despite high vitality, motility remained grossly compromised for the 5.0% DMSO groups. A morphology assessment provided the reason for this observation. This group had the highest increase in tail abnormalities, suggesting that tail coiling is likely responsible for the observed low motility.

This concentration-dependent phenomenon reflects the reduced capacity of CPAs at lower concentrations to protect against irreversible structural damage. In contrast, higher concentrations are more apt to protect cells against damage but are more prone to exert some cytotoxic effects [182, 279–281].

The use of CPAs is vital for cell survival during cryopreservation. However, their presence causes cell osmotic stress due to solute and water movements. During exposure to permeating CPAs, cells will dehydrate and swell as the water re-enters with the CPA. After cooling and warming, CPA removal initially causes an influx of water (sperm cells swell), and then, sperm cells slowly return to their iso-osmotic volume as CPA and water leave [282]. However, the osmotic tolerance of cells is limited. Human sperm can swell to 1.1 times and shrink to 0.75 times their iso-osmotic cell volume without having their motility compromised [201]. Furthermore, cell permeability decreases as the molecular size of the substance increases. For human spermatozoa, glycerol is three times more membrane permeable than DMSO [283, 284]. In the present study, a cryoprotective synergism between glycerol (and sucrose) and DMSO facilitated sperm survival after a freeze-thaw cycle. Similar results, although at different concentrations/combinations of glycerol and DMSO, have been reported for sperm in various species [285, 286]. From a cryobiological perspective, glycerol alone, at low concentrations (<20%, the typical amount used in SFMs [287]), is insufficient to fully prevent crystallisation [283]. As a result, adding 5.0% DMSO may have more effectively delayed ice crystallisation (than 2.5% DMSO), significantly improving sperm survivability.

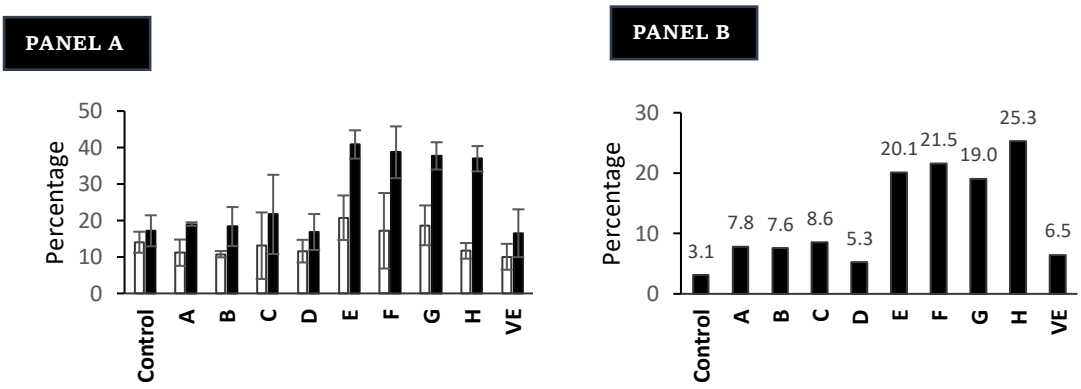

Figure B.7. Total Motility and Vitality for the different experimental groups. Panel A- Post-thaw total motility and vitality by group. Bars represent standard deviation. White – total motility, Black – vitality. VE – vitamin E. Panel B – Percentage of live immotile sperm cells.

However, because DMSO is less permeable to human spermatozoa, it crosses the sperm membrane at a slower rate than glycerol [283, 284]. Therefore, DMSO is more likely to cause osmotic damage at higher concentrations [283, 284]. This fact, we hypothesise, is responsible for the low motility and high percentage of coiled tails obtained throughout the E-H experimental groups, despite the higher vitality and morphology. Gao et al. [288] estimate that human spermatozoa lose close to 60% motility when their volume exceeds their upper osmotic tolerance limit (1.38 times their iso-osmotic volume).

Although not extensively explored, sucrose is another crucial component of the freezing medium. This non-permeating CPA minimises damaging sperm cell volume shifts during cooling and warming. Adding permeating agents in the freezing medium, such as DMSO, ASTX, and VE, further accentuates the importance of this action [282, 289].

DMSO is widely used for cell cryopreservation due to its membrane penetrating and water displacement properties [282]. Furthermore, its vast solubilising capacity means DMSO is frequently employed as a solvent [290]. The use of this solvent is so ubiquitous that, in many studies, DMSO concentration is often unreported. For sperm cryopreservation studies, these preliminary results call for renowned attention to the DMSO concentration used by researchers, as they may exert considerable influence on post-thaw sperm parameters.

4. The Antioxidants

4.1 Vitamin E

In 2011, Kalthur et al. [239] evaluated the effect of vitamin E supplementation in a freezing medium on post-thaw motility and DNA integrity. They found that 5 mM VE exhibited the best protective effect on cryopreserved sperm cells, and normozoospermic samples showed, on average, 56% post-thaw total motility. The obtained results with VE supplementation differ significantly from those reported by Kalthur et al. [239]. This fact speaks not only to the low reproducibility transversal to all sperm cryopreservation studies but also to the importance of factors such as freezing medium constituents, pre-freeze sperm concentration, sperm preparation techniques, and the presence or removal of seminal plasma before freezing in post-thaw semen quality.

Here, the present study tested the protective effect of 5mM VE under particular conditions. The effect of VE supplementation was evaluated (1) in a consistent number of sperm cells (20 million sperm cells/ml), (2) without seminal plasma potential influence, and (3) in a

consistent cryopreservation volume (400 μL). The study by Kalthur et al. [239] failed to report such restrictive conditions in their analysis of VE supplementation. Furthermore, they used a glycerol-egg yolk-citrate medium as a cryopreservation medium, in contrast to the HEPES buffered medium with glycerol, sucrose and human serum albumin used in the present study. Some authors suggest that a medium containing egg-yolk citrate increases post-thaw sperm survival, although consensus is hard to find [291]. Cryopreservation with added egg yolk is believed to increase sperm membrane stability and reduce the deleterious effect of a hyperosmotic environment that occurs during rapid freezing [291, 292].

Our results contradict various studies supporting Kalthur et al. [239] findings. Once again, these are less 'restrictive' studies in which pre-freeze sperm concentration and volume are not elucidated or even mentioned as potential confounders, and different cryopreservation media, cryopreservation protocols and VE concentration were used [291]. Among the studied groups, VE exhibited the lowest performance in terms of motility (10.0%) and vitality (14.0%). However, no statistically significant differences were observed when compared to the control group.

4.2 Astaxanthin

Compared to VE, studies show that ASTX displays more robust antioxidative activity [293, 294]. In particular, ASTX was found to have an antioxidant function of up to 100-500 times more significant than VE [295]. ASTX has been reported to improve sperm quality by decreasing ROS production in seminal plasma. In particular, a study that involved oral supplementation with ASTX in male individuals found a significant reduction in seminal ROS and improvement in sperm motility and morphology [296]. Positive results were also found in other species. In particular, Basioura et al. reported higher viability and motility in frozen-thawed boar semen [297]. Similar positive effects of ASTX on post-thaw semen quality were observed in roosters [298], miniature pigs [299], and ram sperm [300].

Here, the main positive effect of ASTX was on DNA integrity. The combination of 5% DMSO and 15 μM ASTX produced the best protective effect against DNA damage. A study conducted on the DNA binding property of ASTX, both *in silico* and *in vitro*, shows that, by binding to the major and minor grooves, ASTX might protect DNA against oxidative stress [301]. This protective role has been described in *Saccharomyces cerevisiae* [301] and sperm from streptozotocin-induced diabetic rats [302]. Furthermore, in a similar cryopreservation study, the effects of ASTX on sperm from 30 male individuals were evaluated [303]. The authors found that 100μM ASTX was beneficial for chromatin condensation and sperm motility. Once again, a direct comparison is hindered by the fact that the authors do not clarify the volume and concentration of the sperm used or specify the final concentration of DMSO in the freezing medium. Nonetheless, a protective effect on DNA integrity is reported [303]. However, at the lowest tested DMSO concentration (2.5%), ASTX's protective role against DNA damage was not evident. Three main factors combine to damage sperm cells and increase the rate of ROS production during cell cryopreservation: osmotic stress, intracellular and extracellular ice formation and oxidative stress. Here, the presence of 5.0% DMSO in a glycerol-based SFM reduced intracellular ROS levels by preventing intracellular ice formation. Such action, in turn, allowed ASTX at the low concentrations of 15 and 25 μM to exert a more noticeable positive effect on DNA integrity.

Overall, the results suggest that, as in other studies with different antioxidants [304], there may be a synergistic effect worth seeking between DMSO and ASTX. Increasing the concentration of ASTX may prove a beneficial strategy for motility, as Dede & Saylan [303] reported a positive effect of 100 μM ASTX for this parameter. Nonetheless, achieving the ideal formulation that maximises the protective properties of both DMSO and ASTX will require

careful balance, as high concentrations of cryoprotectants are prone to exhibit cytotoxic effects [282].

4.3 ASTX and VE combination

The effectiveness of ASTX and VE as antioxidants has been extensively acknowledged. Thus, it was anticipated that combining these compounds would amplify their activities. ASTX was found to complement the antioxidative effect of VE, preventing oxidative damage in a diabetic rat model [305]. In this *in vivo* study, it was postulated that the presence of both ASTX and VE within the lipid membranes could promote functional complementarity. In the present study, however, the combination of ASTX and VE failed to produce better sperm parameters. The presence of VE in the freezing medium appeared to hinder the protective effect 25 μM ASTX displayed in group G against DNA damage.

Antioxidants (as well as combinations of them) only sometimes produce satisfactory results. High concentrations of antioxidants can drastically move the pendulum from oxidative stress to reductive stress. Over-accumulation of reductants has been reported to be capable of compromising the chromatin integrity of sperm cells and contributing to sDF [306]. Therefore, the results of group H might reflect this double-edge property of antioxidants [224]. Nevertheless, the statistical analysis failed to demonstrate significant differences between Group H and the remaining groups (for all the studied parameters), making it challenging to ascertain or accurately speculate the reasons behind the observed values.

5. Present & Future Challenges

A grave limitation of this work pertains to sample size. Irrespective of the employed protocol, variations in semen freezability and fertility have been observed between men. The mechanisms underlying the different susceptibility to cryoinjury between individuals have not yet been elucidated, but a genetic component [307] and seminal plasma constituents [308] are strongly suggested as influential. Due to this enormous variability in cryodamage displayed by spermatozoa, the sample size becomes crucial for sperm cryopreservation studies. Here, the application of a rigorous screening led to a limited pool of normozoospermic semen samples by carefully eliminating factors that could harm semen sample quality, such as health conditions, lifestyle choices (smoking and alcohol consumption) and abnormal semen parameters. This meticulous approach was undertaken to ensure that the effects of ASTX, VE and DMSO supplementation could be accurately assessed without the interference of potential confounding factors. Furthermore, restrictions related primarily to semen volume and concentration limited the range of ASTX, VE and DMSO concentrations subjected to testing. As a result, the statistical power to detect significant differences among the studied groups was severely hindered, and the generalizability of our findings was impacted.

At present, despite the factors mentioned above, the supplementation of freezing medium with antioxidants is strongly backed by a substantial body of evidence. However, there needs to be more consensus regarding the specific type, combinations, and concentrations of antioxidants to be added. As the understanding of cryobiology processes advances, the current deluge of cryopreservation protocols will narrow to a few highly reproducible and efficient ones [309]. Furthermore, a reinforced technological presence is expected in human reproduction laboratories to reduce operator errors and enable the production of consistent results. In particular, microfluidics and chip technologies promise cell monitoring and environmental control during the addition and removal of CPAs during the freeze-thaw cycle [309, 310]. In addition, nanotechnology is being developed for optimal antioxidant protection and delivery to cells. Preliminary studies claim an improvement in the antioxidant effect by virtue of nanoencapsulation [311]. These strategies, powered by the most recent advances in

cryotechnology, will prompt sperm cryo survival rates to skyrocket and revolutionise cryobiology.

Finally, other approaches to safeguard sperm cells against cryodamage have been gaining attention, ranging from innovative cryoadditives and antifreeze proteins to stress preconditioning of spermatozoa before cryopreservation [186].

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CONCLUDING REMARKS

The social status that fits modern society the best is now childlessness. In pursuit of the most remarkable career, financial success and stability in life, a single person encumbered by family responsibilities has been deemed the ideal [110]. As a result, individuals of reproductive age frequently postpone childbearing [312]. Late childbearing, the dominant trend in Portugal and Europe, demonstrates numerous positive aspects, such as better family functioning, higher family stability, and a more stable economic position for parents [312]. However, mainly because they overestimate the success rate of ART therapies, couples (especially men) are blindsided by the age-related association with an elevated risk of infertility [49, 313].

Understanding the occurrence of (male) infertility in a population is vital. This knowledge allows for societal preparedness, provides guidance to health sciences investigators, and allows better patient counselling and education from health care providers. In this way, the characterisation of male patients subjected to semen analysis provided invaluable information on reproductive trends in the Tras-os-Montes and Alto Douro region, especially in contrast to semen donors. Furthermore, the potential impact of the COVID-19 pandemic on reproductive behaviour was discussed. People who have undergone quarantine and social distancing have commonly reported feelings of depression, irritability, poor mood, fear, guilt, and nervousness [314]. Such circumstances (depression and anxiety) are typically associated with low levels of desire. In addition, the resulting economic fallout (housing insecurity, job loss) and health impact (grieving lost family members, trauma, changes in weight, sleep, and alcohol use) bode significant hardship for future couples and fertility specialists [315] and can lead to further increase in the age of childbearing, in the following years.

Good reproductive health relies on the integration of physical, mental, emotional, and social events [316]. The conducted descriptive study allowed the observation of the potential impact (some) modifiable lifestyle factors could exert on semen parameters. Indeed, existing literature strongly suggests that male fertility (and semen parameters) may not only serve as a biomarker of overall health but could also signal the development of comorbidity and mortality [317]. In particular, male infertility has been associated with an increased risk of prevalent and incident oncologic, cardiovascular, metabolic, and autoimmune diseases [317]. Although the short discretionary lifestyle questionnaire at CHTMAD lacks essential sociodemographic information, it can provide a broad but somewhat vital characterisation of the infertile male population in the Trás-os-Montes and Alto Douro region. Moving forward, a potential goal should concern the analysis of the epidemiological andrology data gathered, and efforts should be made to guarantee a better depiction of the infertile male population of Trás-os-Montes and Alto Douro for the following years. This would require an improved questionnaire, better data repositories that are readily available and can be queried to quantify various aspects of the disease (prevalence, treatment outcomes, time to pregnancy, etc.), and a more efficient infrastructure that allows for a rapid and precise exchange of information and data between patients and clinicians in fertility consultations, and between clinicians and the andrology lab technicians. These data could then be linked to socioeconomic, geographic, environmental, and lifestyle factors to spur new research avenues and treatments.

The development of cryopreservation has had an impact in many fields and, most strikingly, in reproductive medicine. This process made it possible to manage and preserve male fertility. However, despite many efforts, sperm cryopreservation is not yet harm-free. Successful cell storage and preservation depend on the ability to carefully induce and reverse the low-temperature state without incurring cell damage. To protect sperm cells against cryodamage,

distinct strategies have been developed. One strategy relies on neutralising ROS by supplementing the freezing medium with antioxidants.

The developed study, although severely hindered by the low number of semen samples, yielded promising results regarding ASTX's protective effect on genomic integrity. Understanding the interplay between DMSO, ASTX, and their concentrations can be essential for effective sperm cryopreservation strategies and thus merits further investigation.

Finally, recent advances in cryotechnology foreshadow a bright future where automating key delicate processes in cryopreservation is an exciting new possibility. Such features will ensure uniformity and reliability across protocols, allowing the pooling of data for systematic comparative analysis. As a result, the arduous endeavour of developing the "perfect" cryoprotective formulation for human sperm cells will be greatly expedited. This will significantly impact the ART technologies and their success rate.

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APPENDIXES

ORIGINAL ARTICLE, SUBMITTED FOR THE "AUSTIN JOURNAL OF REPRODUCTIVE MEDICINE & INFERTILITY" – UNDER PEER-REVIEW PROCESS.

ASTAXANTHIN SHIELDS SPERM DNA AGAINST FREEZE DAMAGE: A PRELIMINARY STUDY

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Abstract

Sperm cryopreservation is a valuable technique for preserving and managing male fertility. However, the freeze-thaw cycle often induces oxidative stress and damage to sperm cells. Astaxanthin (ASTX), known for its potent antioxidant properties, holds promise in mitigating the harmful effects of oxidative stress.

This study investigated the effects of ASTX and DMSO on post-thaw sperm parameters. Rigorous exclusion criteria were applied in the samples selection to ensure a precise and reliable analysis, eliminating potential confounding factors such as detrimental lifestyle habits, vitamin intake, history of diseases, and abnormal semen parameters. Subsequently, from an initial pool of 150 participants, six carefully selected semen samples were used, and seven experimental groups were established. The control group was comprised solely of sperm freezing medium (SFM), and the remaining groups received SFM supplemented with three different concentrations of ASTX (0, 15, 25 μM), each dissolved in DMSO at concentrations of 2.5% and 5.0%. Sperm motility, vitality, morphology, and DNA damage both before and after freezing were assessed for each group.

Five per cent DMSO was generally more beneficial, with significant improvement in sperm vitality (40.8%) and DNA damage (22.2%), over the 2.5% DMSO and control groups. Moreover, ASTX at 5.0% DMSO displayed the most marked impact on DNA damage. The concentrations of 15 μM and 25 μM resulted in minor increases in DNA damage (7.5% and 12%, respectively). In comparison, the control group exhibited significantly higher values (33.4%). These results highlight the potential of ASTX in mitigating DNA damage during sperm cryopreservation.

Keywords: Sperm; Cryopreservation; Antioxidants; Astaxanthin; Cryoinjury

Abbreviations ASTX: Astaxanthin; DMSO: dimethylsulfoxide; DNA: deoxyribonucleic acid; ROS: Reactive Oxygen Species; SFM: Sperm Freezing Medium.

Introduction

Sperm cryopreservation is a valuable service that allows men to preserve their fertility before undergoing procedures or exposures that could harm their reproductive potential. Additionally, sperm cryostorage is pivotal in cases of severe oligozoospermia (low sperm count), intermittent presence of motile sperm cells in semen, or partially successful infertility treatment. This technique also offers substantial advantages to assisted reproductive technologies [1].

Successful cell cryopreservation depends on the ability to induce and exit the low-temperature state without cell damage. However, the freezing and thawing cycle frequently changes the sperm membrane lipid composition, and acrosome status, severely hinders sperm motility and viability and has been reported to increase sperm deoxyribonucleic acid (DNA) damage [2]. Cell cooling not only causes physical injury but also results in the excessive production of free radicals that disrupt vital chemical structures in the sperm cell. In addition, sperm DNA is especially vulnerable to oxidative damage because the highly condensed nuclear structure of sperm prohibits the enzymatic repair of damaged DNA [3]. Therefore, to maintain functionality, sperm cells undergoing a freeze-thaw cycle need to balance their redox potential [4].

Currently, researchers are exploring various methods to safeguard sperm cells from freeze damage. One strategy relies on neutralising reactive oxygen species (ROS) through freezing medium supplementation with antioxidants. In principle, implementing such action can enhance the antioxidant defence mechanisms of sperm and preserve, to a satisfactory degree, the biological potential of sperm cells post-thaw [5].

Astaxanthin (ASTX) is a marine xanthophyll (oxygenated carotenoid) produced by algal species (*Haematococcus pluvialis*, *Chlorella zofingiensis* and *Chlorococcum*), bacteria, and fungi [6]. This antioxidant has received increasing attention as an effective molecule to counteract and dilute the effects of conditions related to oxidative stress [7]. Numerous studies have been conducted on its anticancer, antidiabetic, anti-inflammatory, immune-stimulating, and antioxidant properties, suggesting a potential therapeutic effect [8-10].

Here, freezing medium supplementation with ASTX was performed at different dimethylsulfoxide (DMSO) concentrations to ascertain the possible beneficial effect on sperm cryopreservation.

Materials and methods

Between March and July 2022, 150 semen samples were collected from men attending fertility support consultations at the Trás-os-Montes and Alto Douro Hospital Centre. A rigorous exclusion criterion was applied in these samples to ensure accurate and reliable data and to eliminate factors that could affect ROS generation and compromise sperm quality. Participants with a history of drug addiction, smoking, alcohol consumption, prolonged diseases, drug use (including vitamins), as well as samples exhibiting abnormal semen parameters (below the threshold set by the World Health Organization [11]) or suspected of leukocytospermia were excluded from the study. Furthermore, samples that did not liquefy within 30-40 minutes of incubation at 37°C or had a volume below 1.5 ml or a sperm concentration less than $20x10^6$ sperm cells/ml were also removed from the study. Consequently, only six out of the initial pool of 150 samples met the strict criteria and were included in the study and could be considered a pool of normal sperm cells.

The six semen samples were distributed into seven groups (A-G) (Table 1). Each sample with an adjusted concentration of $20x10^6$ sperm cells/ml underwent rapid freezing cryopreservation (the cryovials were placed 10 min at room temperature, followed by a 30 min exposure to nitrogen vapours before submersion in liquid nitrogen) with sperm freezing medium (SFM) (ORIGIO, Måløv, Denmark) supplemented with six different concentrations of DMSO and ASTX (Sigma-Aldrich Company, St. Louis, MO) (Table 1): group A (control – SFM only), group B (2.5% DMSO), group C (15 μM ASTX in 2.5% DMSO), group D (25 μM ASTX in 2.5% DMSO), group E (5.0% DMSO), group F (15 μM ASTX in 5.0% DMSO), and group G (25

μM ASTX in 5.0% DMSO). After two weeks, the cryovials were thawed by submersion in a 37°C water bath for 5 min before immediate freezing medium removal. Sperm motility, vitality (Eosin Y 0.5%, Merck) morphology, and DNA damage (Alkaline Comet Assay) were evaluated before freezing and after thawing. DNA damage was assessed through the classification of comets into five categories (visual score) (0-4) according to amount of DNA in tail (100 cells were scored per gel). The obtained visual score, following Azqueta et al. [12], was divided by four and converted to the percentage of sperm DNA in tail.
The normality of data was analysed by the Shapiro-Wilk test. Parameters were compared between groups using One Way-ANOVA and Kruskal-Wallis, followed by post hoc tests. The value of $p < 0.05$ was regarded as statistically significant. The statistical analysis was performed using R Statistical Software (version 4.2.2; R Foundation for Statistical Computing, Vienna, Austria).

Results

The semen samples enrolled in our study exhibited, before cryopreservation, an average volume of 3.0 ml, total motility of 64.5%, 71.6% vitality, sperm count of $45.9x10^6$ sperm cells/ml, 6.0% sperm cells with normal morphology and 25.5% of sperm DNA in tail.
After two weeks of storage, the findings in Table 2 reveal that all the studied parameters were negatively impacted, irrespective of the conditions tested. Regarding motility and morphology, DMSO alone or combined with ASTX yielded no statistically significant improvements over the control group A. Indeed, total motility (progressive and *in situ* movements) varied between 10.1%, group C (15 μM ASTX in 2.5% DMSO), and 20.8%, group E (5.0% DMSO). On average, the groups with 5.0% DMSO alone or in combination with ASTX (E-G) displayed higher values for total motility (20.8, 17.2, 18.7%, for groups E-G, respectively) when compared to groups with 2.5% DMSO (11.2, 10.1, 13.1% for groups B-D, respectively) and control group A (14.0%). When analysing morphology, group D (25 μM ASTX in 2.5% DMSO) exhibited the lowest percentage of normal sperm cells (only 2.5%), while Group F (15 μM ASTX in 5.0% DMSO) showed the highest (5.3%). Once again, better morphology was seen in the 5.0% DMSO groups (5.3, 5.3 and 4.7, for groups E-G, respectively, vs 3.4, 3.8, 4.3 and 2.5 for groups A-D, respectively).
Concerning vitality, compared to the control group A, only group E, with an average of 40.8% vitality, demonstrated a statistically significant improvement in this parameter ($p = 0.021$) (Table 3). Although no statistically significant difference could be detected for the remaining groups, the highest values for sperm vitality can be found in the 5.0% DMSO alone or in combination with ASTX groups (40.8, 38.7 and 37.7% for groups E-G, respectively, vs 17.2, 19, 18.3 and 21.7% for groups A-D, respectively).
Lastly, significant differences were found in sperm DNA damage (Figure 1 and Tables 2 & 3). Group A, the control, showed, in comparison with all the remaining groups, the highest DNA damage with the most significant gain in % of DNA in tail (33.4%), followed by Groups B-E (22.9, 21.0 and 22.5% DNA in tail, respectively), and lastly, by groups F and G (7.51 and 12%, respectively).

Discussion

Sperm cryopreservation is a challenging process. Freezing injury is frequent and impairs sperm function. In agreement with several studies, the freeze-thaw cycle caused severe damage to the analysed sperm cells [13,14]. For human sperm cryopreservation, glycerol is the preferred cryoprotective agent, as, for sperm cells, it is three times more permeable than DMSO [15,16]. In the present study, all samples were cryopreserved in a glycerol-based (concentration not specified by the manufacturer) SFM, and DMSO was added at two different concentrations. Groups B (2.5% DMSO) and E (5.0 DMSO) were created to evaluate the potential impact of DMSO. Glycerol alone, especially at low concentrations (< 20%), as often found in SFMs [17], is insufficient to prevent crystallisation entirely [16]. Indeed, compared to the control group, only 5.0% DMSO significantly improved sperm vitality. The failure of

2.5% DMSO to enhance sperm vitality may be attributed to its reduced capacity to delay ice crystallisation. This observation highlights the limited ability of cryoprotectants at lower concentrations to protect against irreversible structural damage [18]. Similar results, although at different concentrations/combinations of glycerol and DMSO, have been reported for sperm in various species [19,20]. In this study, as standard practice, DMSO was employed primarily as (ASTX) solvent. However, our preliminary findings indicate that the percentage of DMSO (v/v) is crucial, as it can significantly impact sperm parameters. The data obtained reveal a positive effect of 5.0% DMSO supplementation (groups E-G), over the control group, across all the studied parameters, with statistical significance reported only for vitality and DNA damage.

ASTX has been reported to improve sperm quality through oral supplementation by decreasing ROS production in seminal plasma and improving sperm motility and morphology [21]. In mice, ASTX treatment showed a protective effect on sperm DNA against cyclophosphamide-induced damage [22]. Regarding cryopreservation studies, Basioura et al. reported higher viability and motility in frozen-thawed boar semen using ASTX [23]. Similar positive effects of ASTX on post-thaw semen quality were also observed in other species, such as roosters [24], miniature pigs [25], and ram sperm [26]. More importantly, in a recent study, the effects of ASTX on cryopreserved semen samples from 30 males were evaluated [27]. Although not reporting the applied DMSO concentration, the authors found that 100 μM ASTX effectively decreased chromatin damage and improved sperm motility.

Regarding our preliminary results, both tested ASTX concentrations at 5.0% DMSO showed a protective effect against DNA damage. Group F (15 μM ASTX in 5.0% DMSO) and G (25 μM ASTX in 5.0% DMSO) exhibited the lowest increase in DNA in tail, with only 7.5% and 12%, respectively. A study conducted on the DNA binding property of ASTX, both *in silico* and *in vitro*, suggests that ASTX exerts this protective action through binding to the major and minor grooves [28]. However, at the lowest tested DMSO concentration (2.5%), ASTX's protective role against DNA damage was not evident. Three factors combine to damage sperm cells and increase the rate of ROS production during cell cryopreservation: osmotic stress, intracellular and extracellular ice formation and oxidative stress. Here, the presence of 5.0% DMSO in a glycerol-based SFM reduced intracellular ROS levels by preventing intracellular ice formation. Such action, in turn, allowed ASTX at the low concentrations of 15 and 25 μM to exert a more noticeable positive effect on DNA integrity. Thus, the observed low percentage of DNA fragmentation in groups F and G reinforces the reported antioxidative activity of ASTX [22,27].

These initial findings point to a beneficial combined effect of DMSO and ASTX that warrants further exploration. Furthermore, increasing the concentration of ASTX may prove a beneficial strategy for motility, as Dede & Saylan [27] reported a positive effect of 100 μM ASTX for this parameter. Nonetheless, achieving the ideal formulation that maximises the protective properties of both DMSO and ASTX will require careful balance, as high concentrations of cryoprotectants are prone to exhibit cytotoxic effects [2].

Lastly, a limitation of this work concerns sample size. In order to ensure a precise evaluation of ASTX and DMSO supplementation effect, we conducted a meticulous sample selection process. This enabled us to treat the selected samples as a representative pool of normal spermatozoa.

Conclusion

Cryopreservation is a complex procedure that requires the precise regulation of many factors to ensure (minimal) success. The developed preliminary study, although severely hindered by the low number of semen samples, yielded promising results regarding ASTX's protective effect on human sperm genomic integrity. Furthermore,

our findings suggest that 5.0% DMSO in combination with ASTX shows promise in improving sperm vitality and reducing DNA damage. The interplay between DMSO, ASTX, and their

concentrations can be essential for effective sperm cryopreservation strategies and thus merits further investigation.

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Figures and Tables

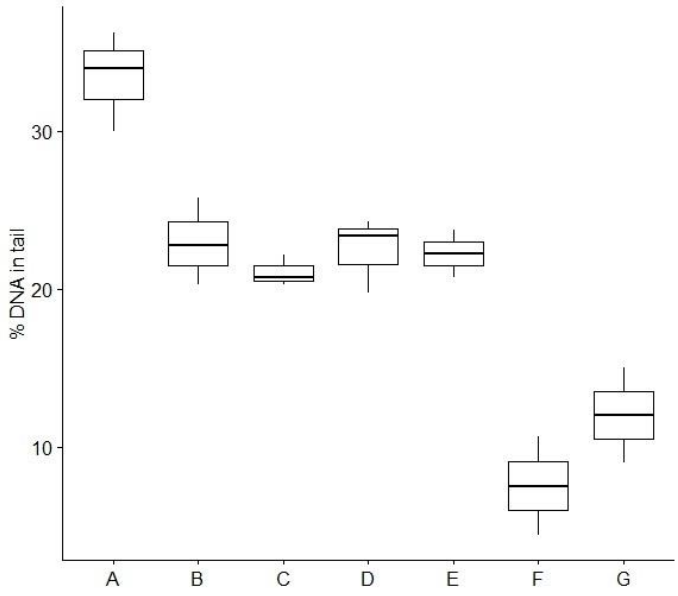

Figure 1. Box-plot showing the effect of freezing medium supplementation on sperm DNA fragmentation after a freeze-thaw cycle. Results are represented as increase (%) in sperm DNA in tail as a function of ASTX and DMSO freezing medium supplementation (experimental groups A-G) after two weeks of storage.

Table 1. Study design.

Groups	**A**	**B**	**C**	**D**	**E**	**F**	**G**
	SFM						
DMSO (%)	-	2.5			5.0		
ASTX (µM)	-	0	15	25	0	15	25

ASTX, astaxanthin; DMSO, dimethylsulfoxide; SFM, sperm freezing medium.

Table 2. Post-thaw comparison of sperm parameters.

	Control	**2.5% DMSO**			**5.0% DMSO**		
ASTX (µM)	-	0	15	25	0	15	25
Groups	A	B	C	D	E	F	G
Parameters (%)							
Total Motility	14.0 ± 2.9	11.2 ± 3.6	10.1 ± 0.9	13.1 ± 9.1	20.8 ± 6.1	17.2 ± 10.4	18.7 ± 5.5
Vitality	17.2 ± 4.3^{b}	19 ± 0.5^{ab}	18.3 ± 5.5^{ab}	21.7 ± 10.9^{ab}	40.8 ± 3.9^{a}	38.7± 7.1^{ab}	37.7 ± 3.7^{ab}
Normal Forms	3.4 ± 1.9	3.8 ± 2.3	4.3 ± 2.4	2.5 ± 0.7	5.3 ± 1.5	5.3 ± 3.2	4.7 ± 0.6
DNA in tail*	33.4 ± 3.2^{a}	22.9 ± 2.6^{b}	21.0±1.0^{bc}	22.5± 2.4^{b}	22.2 ± 2.1^{b}	7.5 ± 4.4^{d}	12.0 ± 4.2^{c}

Note: Data are presented as mean ± standard deviation. Different letters indicate statistically significant differences ($p < 0.05$). * Increase in DNA in tail. ASTX, astaxanthin; DMSO, dimethylsulfoxide; SFM, sperm freezing medium.

Table 3. Obtained p-value for multiple comparisons of means for post-thaw sperm parameters.

	Vitality	**% DNA in tail**
Groups	p-value	
B-A	1.0000	0.0116035*
C-A	1.0000	0.0034462*
D-A	1.0000	0.0085763*
E-A	0.0211*	0.0163227*
F-A	0.0915	0.0000123*
G-A	0.0741	0.0000775*
C-B	1.0000	0.9800033
D-B	1.0000	0.9999926
E-B	0.0667	0.9999649
F-B	0.2913	0.0014431*
G-B	0.2362	0.0189624*
D-C	1.0000	0.9952487
E-C	0.0540	0.9989200
F-C	0.2362	0.0040701*
G-C	0.1915	0.0587698
E-D	0.0600	1.0000000
F-D	0.2623	0.0018494*
G-D	0.2127	0.0249874*
F-E	1.0000	0.0042519*
G-E	1.0000	0.0486060*
G-F	1.0000	0.7028412

*p-value < 0.05

Cytogenetic Findings In Infertile Couples From Trás-os-Montes And Alto Douro Region: A Glimpse Into The Genetic Basis Of Infertility

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Introduction: A major contributing cause of infertility is chromosomal abnormalities. The production of unbalanced gametes during meiosis leads to a history of recurrent pregnancy loss or adverse delivery outcomes. Thus, it is not surprising that the prevalence of chromosomal abnormalities is 10 to 15 times higher amongst infertile couples. Our study aims to determine the incidence and patterns of chromosome abnormalities among infertile couples from the Trás-os-Montes and Alto Douro Region.

Methodology: Peripheral blood lymphocyte karyotype analysis was performed on 883 patients who attended the Trás-os-Montes and Alto Douro medical center for infertility consultation between January 2010 and July 2022. Karyotyping was done by conventional cytogenetics.

Results: Chromosomal abnormalities were found in 24 couples (2,7%), 11 female (1,2%) and 13 male (1,5%) partners. Balanced translocations, all involving autosomes, were the most common aberration (41,7%). Sex chromosome aneuploidies (37,5%), the presence of marker chromosomes (12,5%), and inversions (8,3%) were next in frequency. Five patients exhibited low-grade mosaicism.

Discussion: The incidence of chromosomal anomalies in infertile couples varies considerably, country and region-wise, with studies describing rates ranging from 1,3 to 15%. For the sampled region, we report a 2,7% incidence of chromosomopathies in infertile couples, nine times the estimated birth rate of chromosome abnormalities assigned to the European population. Our research highlights the significance of chromosomal structural and numerical aberrations and their associated effect on reproduction. Karyotype analysis, despite its known drawbacks, is essential to the diagnostic process. The presence of cytogenetic abnormalities allows for proper counseling and management of the affected couple.

CYTOGENETIC FINDINGS IN INFERTILE COUPLES FROM TRÁS-OS-MONTES AND ALTO DOURO REGION

A Glimpse Into The Genetic Basis Of Infertility

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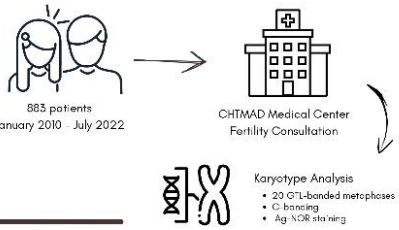
01 INTRODUCTION

Infertility is the failure to conceive after one year of regular unprotected intercourse. The causes can be genetic, environmental, or lifestyle related. The genetic component consists of single-gene anomalies, epigenetic aberrations, and chromosomal (numerical or structural) anomalies [1,2]. For the latter, the known incidence is 10 to 15 times higher in infertile couples [1]. Such a finding is not unexpected: unbalanced gametes produced during meiosis lead to a history of recurrent pregnancy loss or adverse delivery outcomes. As such, karyotype analysis is a crucial technique for fertility studies.

Here we report the incidence and patterns of chromosome abnormalities in infertile couples from Trás-os-Montes and Alto Douro Region over a 12-year period.

02 METHODOLOGY



03 RESULTS

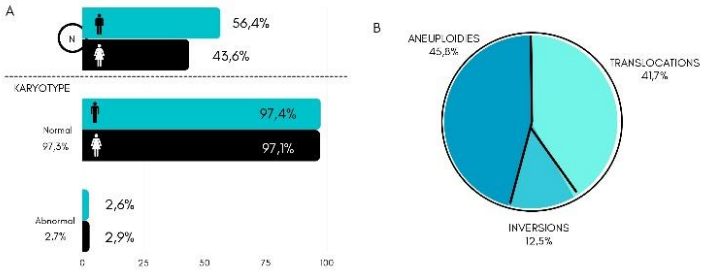


Figure 1. Summary of cytogenetic findings. **A** - Sample distribution by gender and summary of the karyotype analysis performed in 883 patients; **B** - Type and frequency of cytogenetic anomalies in the sampled population .

Table 1. Cytogenetics Findings in Infertile Couples by Gender

♂	♀
47,XXY	45,X
47,XYY	45,X[7]/47,XXX[4]/46,XX[95]
46,XY,ins(4;22)(p16.1;q11)	46,XX,t(5;9)(q13;q34)
46,XY,t(4;10)(q35.1;q25)	46,XX,t(10;15)(q11.23;q13)
46,XY,-(6;8)(p23;q21.3)	46,XX,t(12;15)(q13.3;q24.3)
46,XY,r(8;17)(p22;q21.32)	46,XX,t(2;15)(p22;q12.1;q26.2)
46,XY,r(11;22)(q14.2;q13.1)	46,XX,inv(3)(q21q25)
45,XY,ser(13;14)(q10;q10)	46,XX,inv(14)(q13q24.3)

Figure 2. Autossomes vs Sex chromosomes frequency in cytogenetic abnormalities found.

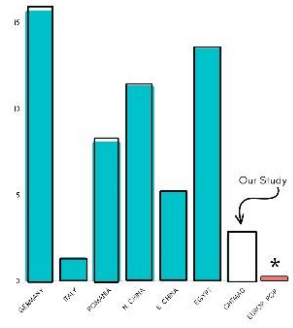


Figure 3. Incidence (%) of chromosomal anomalies in infertile couples, country, and region-wise. * - incidence of cytogenetic anomalies in the European population, in general.

04 DISCUSSION

The incidence of chromosomal anomalies in infertile couples varies considerably, country and region-wise, with studies describing rates ranging from 1,3 to 16% [3-8]. For the sampled region, we report a 2,7% incidence of chromosomopathies in infertile patients [9]. Additionally, we found that infertile females were more prone to chromosomal abnormalities (2,9% vs 2,6% in males). Although not statistically relevant, this finding is in line with several studies [4,8].

Differences in sample size, ethnicity, and selection bias (most of the previous studies were carried out in specific infertile groups: couples undergoing ICSI, receiving assisted reproductive techniques, and couples with reproductive diseases) may explain our (comparatively) low frequency of chromosomal aberrations. Markedly, the present study was conducted in a non-selected infertile population. For this reason alone, we believe, the type and frequency of chromosomal anomalies in the sampled infertile population may be more properly reflected. Furthermore, and supporting our findings, a similar retrospective study (n=2078) conducted in Portugal by Fernandes *et. al* (2022) [10] found a 2,4% incidence of chromosome abnormalities in infertile couples.

Our research highlights the significance of chromosomal structural and numerical aberrations and their associated effect on reproduction. The presence of chromosomopathies enables proper counseling and management of the affected couple. Thus, the significant role played by conventional cytogenetics alone in infertility studies.

05 REFERENCES

The Challenge of Cryopreservation for Male Fertility Preservation

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The development of cryopreservation techniques has had an impact in many fields and most strikingly in Reproductive Medicine. This process has made possible the management and preservation of male fertility. However, sperm cryopreservation is not harm-free. The successful storage and preservation of cells are dependent on the ability to induce and reverse the low temperature state. Cell damage frequently occurs by freezing injury and has two recognizable components: direct damage from the ice crystals and secondary damage due to an increase in solute concentration. Cryoprotectants act by reducing the amount of ice that is formed, but in high concentrations, osmotic and toxic damage are the result, dramatically decreasing the sperm fertility potential.

This review focuses on describing the detrimental effects of cryopreservation on sperm cells and discusses the most recent strategies been implemented to protect sperm cells against cryodamage: from novel cryoadditives, antifreeze proteins, and antioxidants, to stress preconditioning of spermatozoa before cryopreservation.

Keywords: Infertility, Cryopreservation, Sperm

The Breaking Point: Impact of Reciprocal Translocations in Male Infertility

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Keywords: Andrology, Male infertility; Cytogenetics; Reciprocal Translocations.

Chromosomal abnormalities are an important etiologic factor regarding male infertility. The frequency of numerical or structural defects in infertile males varies from 2,2 to 15,2%, in which sex chromosome abnormalities and Robertsonian translocations are the most common. Reciprocal translocations have a much lower incidence and are associated with low sperm count (oligospermia) or even absence of sperm (azoospermia). Azoospermia is to be expected if sex chromosomes are involved, while oligospermia is more frequent in translocations involving only autosomes. Spermatogenesis in these cases is believed to be impaired by two main processes: damage of critical genes to spermatogenesis in the breakage and recombination of chromosome translocation, and through spermatogenetic arrest. However, normal semen parameters can still be found in such carriers. Chromosomal breakpoints seem to dictate whether the male infertility is pregestational (failure to produce a fertilized ovum), gestational (embryo loss after fertilization) or both.

Here we report four different cases of male infertility involving autosome-only reciprocal translocations with different semen profiles. Three male patients with a t(4,22)(p16.1;q11), t(4,10)(q31.3;p15) and a t(11,22)(q14.2;q13.1) with altered semen parameters and a t(6,8)(p23;q21.3) with normal semen profile.

All the above four translocations have been previously associated with male infertility. And, although with different breakpoints in play, t(4,22) and t(6,8) have been described in patients with normal semen profile but with gestational infertility; t(11,22) has been reported, so far, only in oligozoospermic males and t(4,10) exhibits both forms of infertility. Regarding the discovered breakpoints, we found three associated with infertility: 4p16.1 with pregestational infertility, 4q31.3 with gestational infertility, and 10p15 with both. Uncovering these delicate associations between translocations breakpoints and male infertility can provide invaluable insights into the process of spermatogenesis.

THE BREAKING POINT:

IMPACT OF RECIPROCAL TRANSLOCATIONS IN MALE INFERTILITY

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01 INTRODUCTION

Chromosomal abnormalities are an important etiologic factor in male infertility. The frequency of numerical or structural defects in infertile males varies from 2,2 to 15,2% in which sex chromosome abnormalities and Robertsonian translocations are the most common[1,2].

Reciprocal translocations have a much lower incidence[3], but they can hinder spermatogenesis through damage of critical genes and spermatogenetic arrest[4]. As such, absence of sperm in ejaculate is to be expected if sex chromosomes are involved and low sperm count in translocations involving only autosomes[5].

Chromosomal breakpoints seem to dictate whether the male infertility is pregestational (failure to produce a fertilized ovum) or not[6].

MALE INFERTILITY AFFECTS 7% OF ALL MEN[7]

15% GENETIC CAUSES:

- 7% Sex chromosomes abnormalities;
- 1 – 3% Robertsonian Translocations
- **0,9% Reciprocal Translocations**

02 METHODOLOGY

Males who attended infertility consultation at the Hospital Center of Trás-os-Montes e Alto Douro were subjected to a semen analysis (performed according to World Health Organization (2010) guidelines) and cytogenetic study : blood cultures, GTL, and CBL bands were conducted following the established laboratory protocols. Chromosome analysis of at least 20 metaphases was performed (additional 15 metaphases, when necessary, were evaluated to exclude mosaicism).

03 RESULTS & DISCUSSION

We present four different cases of male infertility associated with reciprocal translocations, involving only autosomes, and their respective semen profile. All the following translocations have been previously reported and associated with male infertility.

Patient 1, 46, XY, t(4;22)(p16.1;q11) exhibited low sperm concentration (oligospermia) and low sperm motility. Reports of two patients with the same translocation can be found, although with different breakpoints and a normal semen profile[8,9]. The breakpoint 4p16.1 has been linked to pregestational infertility[10].

Patient 2, 46, XY, t(4,10)(q31.3;p15) had low sperm concentration and high percentage of sperm with morphological abnormalities (oligoteratozoospermia). The translocation has been associated in other fourteen cases with oligospermia and miscarriages [9,11]. The 10p15 breakpoint is the only one found to be associated with impaired spermatogenesis[11].

Patient 3, 46,XY, t(11,22)(q14.2;q13.1) exhibited oligoteratozoospermia. Literature describes one case of male infertility due to this translocation and with a similar semen profile[8].

Patient 4, 46, XY, t(6,8)(p23;q21.3), despite the history of infertility, had normal semen parameters. This agrees with reports of two other cases where the same translocation (different breakpoints) had no apparent effect on spermatogenesis[8,12].

Studies reveal that almost 60% of reciprocal translocations carriers exhibit a normal semen profile[13]. For such cases, a natural conception is still a valid option (reported natural live birth rate for translocation carriers is 65-83% cumulatively[14]). However, these patients are at increased risk of implantation failure and miscarriages. As such, appropriate counseling is paramount for the selection of suitable reproductive options.

Furthermore, uncovering the association between translocations breakpoints and male infertility can provide invaluable insights into the process of spermatogenesis. Especially when the genetic etiology in 40% of male infertility cases remains unidentified[15].

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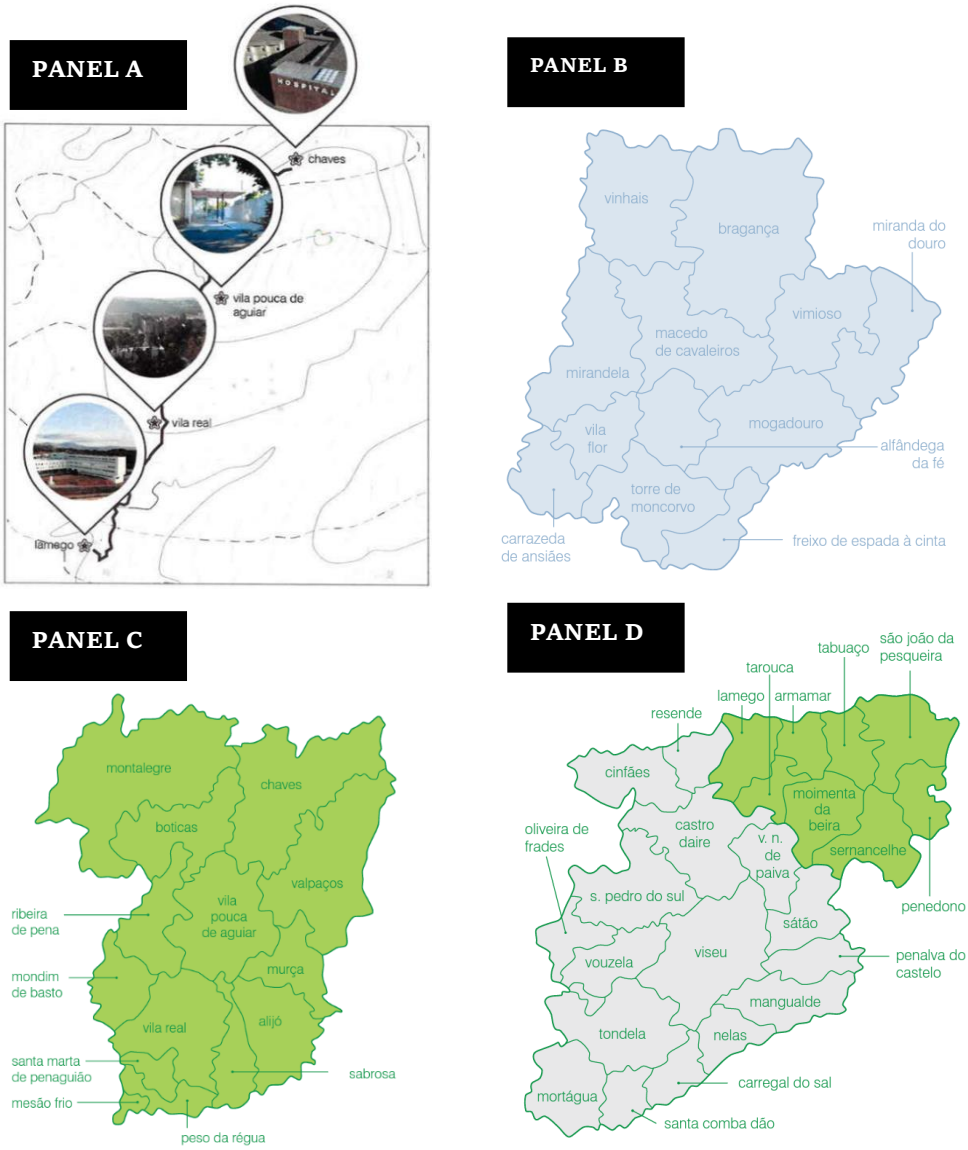

Figure 1. The CHTMAD. Panel A illustrates the four hospital units that comprise the CHTMAD. Panels B through D show the districts of Bragança, Vila Real and Viseu, respectively. In green, CHTMAD's area of direct referral, and pale blue areas of "attraction". The CHTMAD provides health care services to a geographic area of 12,230 km^2, covering 13,2% of the national territory. Source: CHTMAD (2023)[57].

Nº:_____ Idade:_____ Profissão:_____

Data: ___ / ___ / ___

Autorizo que a minha amostra (não identificada) seja utilizada para fins de investigação: ☐ SIM ☐ NÃO

Hábitos Tabágicos (por dia)

- 0 cigarros ☐
- 5 a 10 cigarros ☐
- 10 a 20 cigarros ☐
- + que 20 cigarros ☐

Hábitos Etílicos

- 1 copo dia ☐
- 2/4 copos dia ☐
- +4 copos dia ☐

Patologias ☐

- Diabetes ☐
- Insuficiência Renal ☐
- Herpes ☐
- Rubéola ☐
- Toxoplasmose ☐
- Sífilis ☐
- D. Cardíacas

Outro_____

Alimentação

	2	3	4	5	+6
Nº de refeições (dia)	☐	☐	☐	☐	☐
Carnes vermelhas (semana)	☐	☐	☐	☐	☐
Vegetais (semana)	☐	☐	☐	☐	☐
Cereais (semana)	☐	☐	☐	☐	☐
Fruta (semana)	☐	☐	☐	☐	☐

Teve nos últimos 30 dias alguma infecção (urinária, respiratória ou outra...)?

Sim ☐ Não ☐

Qual? _____

Índice de massa corporal

Peso _____

Altura _____

Exercício Físico

	1/2xsem	+2xsem
Caminhada	☐	☐
Futebol	☐	☐
Ginásio	☐	☐

Outro: _____

Ambiental

Contacto com:

- Herbicidas/Pesticidas ☐
- Tintas ☐
- Fontes de calor ☐
- Radiações ionizantes ☐

Outro:_____

Medicação Regular

Anti-inflamatórios _____ Analgésicos _____

Antibióticos_____ Outro _____

Dias de abstinência sexual _____

A amostra foi colhida na totalidade? ☐ SIM ☐ NÃO

chtmad

(Autocolante identificativo)

CONSENTIMENTO LIVRE E ESCLARECIDO
PARTICIPAÇÃO EM ESTUDOS CLÍNICOS OU EPIDEMIOLÓGICOS

IDENTIFICAÇÃO DO ESTUDO
(preencher em português)

Confirmo que expliquei ao participante, de forma adequada e compreensível, a investigação referida, os benefícios, os riscos e possíveis complicações associadas à sua realização.
Informação escrita em anexo: ☐ Não ☒ Sim (n.º de páginas: 4).

Investigador Responsável:

Nome completo: FERNANDA CARINA MONTAGNE LI
(LETRA DE IMPRENSA)

Assinatura: Fernanda Carina Li BI/CC n.º: 14182713

DECLARAÇÃO DO PARTICIPANTE:

- ✓ Compreendi a explicação que me foi fornecida acerca da minha situação clínica e da investigação que se tenciona realizar, bem como do estudo em que serei incluído;
- ✓ Compreendi os objetivos, métodos, benefícios previstos, riscos potenciais do estudo e o eventual desconforto;
- ✓ Autorizo que a informação e dados iconográficos respeitantes a mim sejam partilhados, de forma anónima, com a comunidade científica;
- ✓ Tive oportunidade de fazer todas as perguntas que julguei necessárias, tendo obtido resposta
- ✓ satisfatória;
- ✓ Compreendi que tenho o direito de recusar em qualquer momento a participação no estudo, sem necessidade de dar qualquer explicação e sem que isso possa ter como efeito qualquer prejuízo na assistência que me é prestada, sendo assegurada a confidencialidade da identidade e registos associados.
- ✓ Declaro não ter sido incluído em nenhum outro projeto de investigação nos últimos três meses.
- ✓ Após o período de reflexão, concordo com a participação neste estudo, de acordo com os esclarecimentos que me foram prestados, como consta neste documento, do qual me foi entregue uma cópia.

Nome do participante: .....

Assinatura do participante: .....

BI/CC Nº: .....

Data: _____/_____/_____

Por favor confirme se todos os campos foram devidamente preenchidos e assinados.

(Autocolante identificativo)

Consentimento Informado, Livre e Esclarecido para participação em projeto de investigação sobre o Estudo dos Efeitos da Criopreservação em Parâmetros Espermáticos, Integridade do DNA e Atividade Mitocondrial.

Este documento, designado Consentimento, Informado, Livre e Esclarecido, contém informação importante em relação ao estudo para o qual foi abordado/a, bem como o que esperar se decidir participar no mesmo. ***Leia atentamente toda a informação aqui contida****. Deve sentir-se inteiramente livre para colocar qualquer questão, assim como para discutir com terceiros a decisão da sua participação neste estudo. Se achar que algo está incorreto ou que não está claro, não hesite em solicitar mais informações. Se concorda com a proposta que lhe foi feita, queira rubricar e assinar este documento.*

Título do estudo: ESTUDO DOS EFEITOS DA CRIOPRESERVAÇÃO EM PARÂMETROS ESPERMÁTICOS, INTEGRIDADE DO DNA E ATIVIDADE MITOCONDRIAL
Responsáveis pelo estudo: Fernanda Li (mestranda); Rosário Pinto-Leite (orientadora).
Instituição de Acolhimento: Laboratório de Genética/Andrologia do Centro Hospitalar de Trás-os-Montes

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O estudo para o qual é convidado a participar decorre no Laboratório de Genética/Andrologia do Centro Hospitalar de Trás-os-Montes e conta com a colaboração de instituições como a Universidade de Trás-os-Montes e Alto Douro e a Faculdade de Medicina da Universidade de Coimbra.

Qual o objetivo do estudo? Avaliar os efeitos lesivos da criopreservação e o efeito protetor de certos antioxidantes sobre a criopreservação de espermatozoides; Refinar protocolos de criopreservação atualmente praticados em Reprodução Medicamente Assistida;

Qual a duração e o local de participação? A participação será concretizada com a doação de uma amostra de sémen, colhida em casa, em recipiente próprio e a fornecer pelo grupo de investigação, respeitando o seguinte período de abstinência sexual: 2-5 dias.

Quais os procedimentos em que vou participar? Preenchimento de um pequeno questionário e produção de uma amostra de sémen por masturbação. O transporte da amostra até ao Laboratório de Genética/Andrologia fica da inteira responsabilidade da equipa de investigação.

(Autocolante identificativo)

A minha participação é voluntária? A sua participação é voluntária e pode recusar-se a participar. Caso decida participar neste estudo é importante ter conhecimento que pode desistir a qualquer momento, sem qualquer tipo de consequência para si.

Quais os riscos e benefícios da participação no estudo? Não existem quaisquer riscos na realização do estudo para os participantes. Como agradecimento aos participantes, será emitido um relatório e enviado por email, caso assim o pretendam, sobre a qualidade da amostra produzida (relatório citomorfobioquímico do sémen).

Quem assume a responsabilidade se algo correr mal? Não estão previstos riscos associados a esta investigação.

Como é assegurada a confidencialidade dos dados? A privacidade e confidencialidade dos dados do estudo serão assegurados através de anonimização da amostra produzida e de arquivo em local seguro, nas instalações do Laboratório de Genética/Andrologia do CHTMAD. Apenas os investigadores terão acesso às informações disponibilizadas.

O que acontece aos dados quando a investigação terminar? Os dados serão guardados até 2 anos após publicação dos mesmos, caso haja dúvidas ou resultados que necessitem de reconfirmação.

Como irão os resultados ser divulgados e com que finalidades? O principal objetivo deste estudo e sua divulgação é o de esclarecer e identificar o papel dos antioxidantes na proteção dos espermatozoides contra os efeitos lesivos da criopreservação. A divulgação dos resultados finais do trabalho será feita a partir da dissertação pública da tese, participação em reuniões científicas e através da publicação em revistas com revisão por pares.

(Autocolante identificativo)

Quem devo contactar em caso de dúvidas?

Fernanda Li: **fernanda_carina_ly@hotmail.com** – 935489812

Dra. Rosário Pinto-Leite – Diretora do Lab. Genética/Andrologia do CHTMAD: **MLLEITE@chtmad.min-saude.pt**

Obrigada pela sua participação!

…… Fernanda Carina Li ……

(Fernanda Carina Li)

…… ……

(Dra. Rosário Pinto Leite)

***Declaro ter lido e compreendido este documento**, bem como as informações verbais que me foram fornecidas pelas pessoas que acima assinam. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. Desta forma, aceito participar neste estudo e permito a utilização dos dados que de forma voluntária forneço, confiando em que apenas serão utilizados para esta investigação e nas garantias de confidencialidade e anonimato que me são dadas pela investigadora.*

Nome: …… ……

Assinatura: …… ……

Data: …… /…… /……..

A. MOTILITY

1. Preheat one slide and two coverslips;
2. After homogenising the sample, pipette 10 µL onto the slide and cover with the pre-warmed coverslip;
3. Allow the sample to stabilise and then observe under a phase contrast microscope at 400x magnification;
4. Score 200 spermatozoa into at least five distinct fields;
5. Repeat the procedure from step 2;
6. If the difference between replicates is acceptable, report the percentage of sperm in each category; if the difference is not acceptable, repeat the procedure.

B. VITALITY

Eosin Y 0,5%:

1. After homogenising the sample, mix 10 µL of 0,5% Eosin Y into 10 µL of sample in a microtube;
2. Wait for 5 minutes;
3. Perform a smear with 10 µL of the mixture;
4. After drying, observe under a bright field microscope at 400x magnification;
5. Sort 200 spermatozoa in at least five distinct fields;
6. Repeat the procedure from step 3;
7. If the difference between replicates is acceptable, report the average percentage of viable (green) sperm in the sample; if not, repeat the procedure.

Eosin Y 0,5% solution:

-Sodium Phosphate Buffer: weigh 0,8 g of sodium dihydrogen phosphate p.a. (NaH_2PO_4) and dissolve in 84 mL of distilled water.
-Potassium Phosphate Buffer: weigh 0,8 g of potassium phosphate dihydrogen phosphate (KH_2PO_4) and dissolve in 88 mL of distilled water.

1. Weigh 0,1 g of Eosin Y;
2. Add 18 mL of sodium phosphate buffer;
3. Add 2 mL of potassium phosphate buffer;
4. Mix thoroughly;
5. Store stock solution at room temperature.

HOS Test

1. After homogenising the sample, mix 10 µL of the sample in 90 µL of Hypoosmotic Solution in a microtube.
2. Place in the oven at 35ºC for 30 minutes;
3. Place 10 µL of the solution on a slide and cover it with a coverslip;
4. After 1 minute, once drifting has stopped, observe under a phase contrast microscope with 400x magnification;
5. Score 200 spermatozoa in at least five distinct fields;
6. Repeat the procedure from step 3;
7. If the difference between replicates is acceptable, report the average percentage of viable spermatozoa (coiled tail) in the sample; if not acceptable, repeat the procedure.

Hypoosmotic Solution:
1. Weigh 1,837 g sodium tricitrate ($Na_3C_6H_5O_7.5,5H_2O$);
2. Weigh 3,377g fructose ($C_6H_{12}O_6$);
3. Place in a 250 mL flask;
4. Add 250 mL of distilled water and mix very well;
5. Filter through a 0,2 µm millipore filter into a sterile container;
6. Store stock solution at 5ºC.

C. MORPHOLOGY
1. Perform a smear with the sample;
2. Allow to dry for at least 4 hours;
3. Immerse the slide in methanol for 10 seconds;
4. Allow to dry for 5 minutes;
5. Immerse the slide in Papanicolaou stain (Merck) for 15 minutes;
6. Rinse the slide under running water;
7. Immerse the slide in Shorr's stain (Merck) for 5 minutes;
8. Rinse under running water;
9. Immerse the slide in methanol for 10 seconds;
10. Let the slide dry. Seal with Entelan (Merck) mounting medium;
11. Proceed to the morphological classification of the spermatozoa in a bright field microscope at 1000x magnification (Table 1). Count the normal spermatozoa and the abnormalities in the head, midpiece, tail and/or cytoplasmic residues in 200 spermatozoa. Repeat the count;
12. If the difference between replicates is acceptable, report the average percentage of morphologically normal spermatozoa and the observed abnormalities.
13. Calculate the Teratozoospermia Index.

D. SPERM COUNT
1. Mount the Neubauer chamber (BLAUBRAND®);
2. In a microtube, perform the desired dilution (Table 2) (first add the volume of fixative and then the corresponding sample volume);
3. Vortex the mixture for 15 seconds and pipette 10 µL into one end of the Neubauer chamber;
4. Vortex the mixture again and pipette 10 µL into the other end of the Neubauer chamber;
5. Place the Neubauer chamber in a humidity chamber for 15-20 minutes at room temperature;
6. Count the number of complete spermatozoa in the central grid (Figure 1) under a phase contrast microscope at 400x magnification;
7. If the difference between the two counts is acceptable, proceed with the calculation of the concentration of spermatozoa per ml of ejaculate; otherwise, the counts must be performed again

Fixative
1. Weigh 25 g of Sodium hydrogen carbonate ($NaHCO_3$);
2. Measure out 5 mL of 3.7-4% formaldehyde;
3. Put everything into a 500 mL flask and add distilled water;
4. Store stock solution at 5ºC;

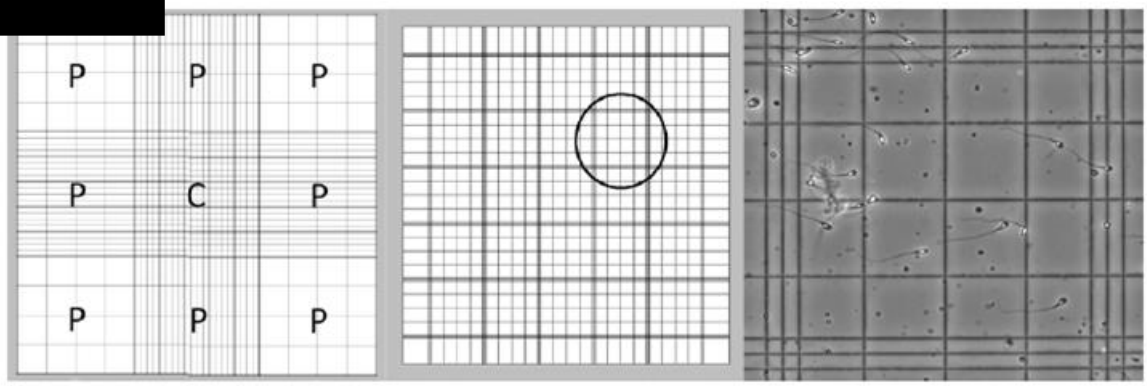
Table 1. Classification of sperm morphology. Source: WHO (2010, 2021)[30, 62]

Location	Normal (ideal/typical) appearance	Abnormal
Head	The head should be smooth, regularly contoured and generally oval in shape. There should be a well-defined acrosomal region comprising 40–70% of the head area (96). The acrosomal region should contain no large vacuoles, and not more than two small vacuoles, which should not occupy more than one fifth of the sperm head. The post-acrosomal region should not contain any vacuoles.	• acrosome less than 40% or larger than 70% of a normal head area, or • length-to-width ratio less than 1.5 (round) or larger than 2 (elongated), or • shape: pyriform (pear shaped), amorphous, asymmetrical, or non-oval shape in the apical part, or • vacuoles constitute more than one fifth of the head area or located in the post-acrosomal area, or • double heads, or • any combinations
Midpiece	The midpiece should be slender, regular and about the same length as the sperm head. The major axis of the midpiece should be aligned with the major axis of the sperm head.	• irregular shape, or • thin or thick, or • asymmetrical or angled insertion at head, or • sharply bent, or • any combinations
Tail	The principal piece should have a uniform calibre along its length, be thinner than the midpiece and be approximately 45 µm long (about 10 times the head length). It may be looped back on itself, provided there is no sharp angulation indicative of a broken flagellum.	• sharply angulated bends, or • smooth hairpin bends, or • coiled, or • short (broken), or • irregular width, or • multiple tails, or • any combinations
Cytoplasmic residue	Cytoplasmic droplets (less than one third of a normal sperm head size) are normal.	• residual cytoplasm is considered an anomaly only when it exceeds one third of normal sperm head size

Table 2. Volumes of ejaculate and fixative (dilution) for adequate sample handling and sperm count. Source: WHO (2010, 2022)[30, 62]

Spermatozoa per ×400 field	Spermatozoa per ×200 field	Dilution	Ejaculate (µl)	Fixative (µl)
> 200	> 800	1 : 50 (1 + 49)	50	2 450
40–200	160–800	1 : 20 (1 + 19)	50	950
16–40	64–160	1 : 10 (1+ 9)	50	450
2–15	8–64	1 : 5 (1 + 4)	50	200
< 2	< 8	1 : 2 (1 + 1)	100	100

PANEL A



PANEL B

Dilution	Number of *large squares* counted in each chamber			Number of *grids* counted in each chamber							
	5	10	25	2	3	4	5	6	7	8	9
	Correction factor values										
1 : 2	*20*	*40*	*100*	200	300	400	500	600	700	800	900
1 : 5	*8*	*16*	*40*	80	120	160	200	240	280	320	360
1 : 10	*4*	*8*	*20*	40	60	80	100	120	140	160	180
1 : 20	*2*	*4*	*10*	20	30	40	50	60	70	80	90
1 : 50	*0.8*	*1.6*	*4*	8	12	16	20	24	28	32	36

Figure 1. Sperm concentration assessment. Panel A – Illustration of the haemocytometer chambers. All nine grids (left) present in one chamber have the same area, but the sizes and numbers of the smaller rectangles vary - the central grid (C) and eight peripheral grids (P). Each grid holds 100 nL. The central grid consists of 25 large squares (middle). A filled chamber (right) shows one of the 25 squares of the central grid containing 16 smaller squares. Panel B. Sperm concentration assessment after sperm count using a haemocytometer. Source: WHO (2010, 2022)[30, 62].

E. SPERM AGGREGATION AND AGGLUTINATION

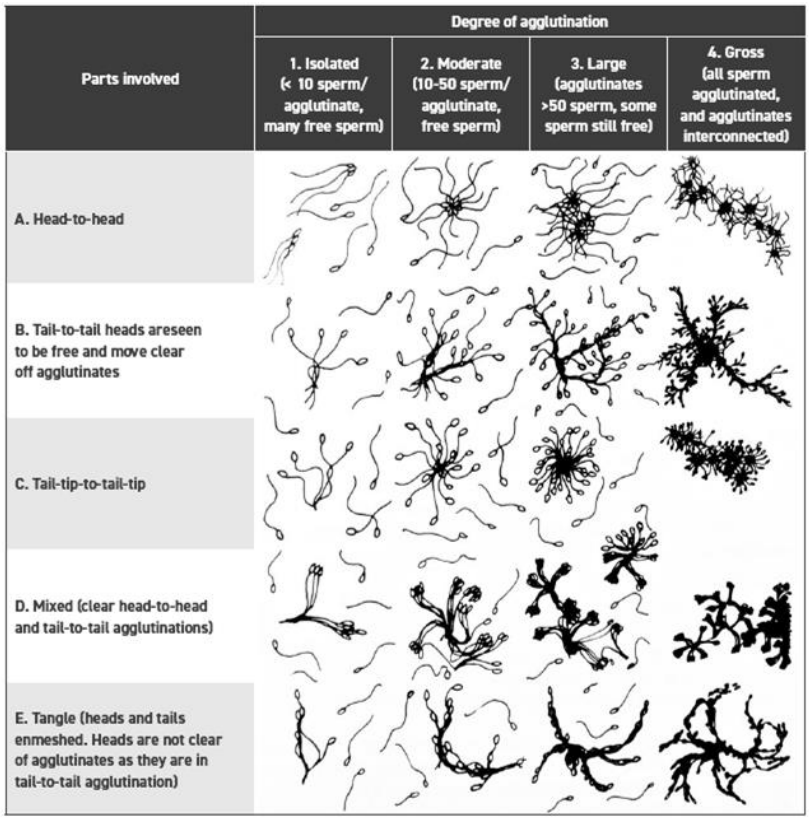


Figure 2. Diagram of different extents of sperm agglutination. Source: WHO (2022)[62].

1. Precoat the slides (Labbox, SLIG-010-050):

1.1. Place a falcon with Normal Melting Point agarose (1% in distilled water) in a 55ºC water bath;

1.2. Dip a slide in the falcon, remove it, clean it underneath and let it dry for at least 24 hours.

2. Suspension of cells in agarose

2.1. Place Low Melting Point Agarose (1% in PBS 1x) in a 37ºC water bath to melt the agarose;

2.2. Place 500 µL of PBS 1x in a microtube;

2.3. Place in the microtube enough sample volume to yield $3x10^4$ sperm cells per 70 µL of gel;

2.4. Centrifuge at 1500 g, 10 minutes;

2.5. Remove the supernatant;

2.6. Add 280 µL of LMP agarose to the pellet;

2.7. Homogenise without bubbling and briefly dispense 70 µL twice onto each slide;

2.8. Immediately place a 20 x 20 coverslip over each drop;

2.9. Maintain at 4ºC, during 5 min;

2.10. Remove the coverslip.

3. Cell Lysis

3.1. Fill a coplin with 40 mL of Base Lysis Solution (2.5 M sodium chloride (NaCl); 0.1 M ethylenediaminetetraacetic acid (EDTA); 10 mM Tris Base and 8 M sodium hydroxide (NaOH) (to adjust pH to 10.0), and add 400 µL of Triton X and 1 mL dithiothreitol (DTT) (0.01 g/mL)- Lysis Solution I;

3.2. Place slides in Lysis Solution I for 1 hour at 4ºC

3.3. Place 40 mL of Base Lysis Solution into a Coplin and add 400 µL of Triton X and 100 µL (20 mg/mL) of Proteinase K (Macherey-Nagel 100 mg)- Lysis Solution II;

3.4. Place slides in Lysis Solution II for 1 hour at 4ºC.

4. Alkaline Treatment and Electrophoresis

4.1. Place the slides in the electrophoresis chamber (Cleaver, Scientific Ltd, CS-250V);

4.2. Fill the chamber until the slides are covered with a thin layer of Electrophoresis Solution (1,2L: 36 mL of 10M NaOH; 12 mL EDTA 0,1 M and distilled water);

4.3. Incubate the sides in the chamber for 30 minutes, with no electrical current, at 4ºC;

4.4. Proceed to electrophoresis at 300 mA, 17 V, 30 minutes.

5. Neutralisation

5.1. Wash slides in PBS 1x in a Coplin, 10 minutes, at 4ºC;

5.2. Wash the slides in distilled water in a Coplin for 10 minutes at 4ºC;

5.3. Remove the slides and leave them to dry upright.

6. Visualisation

6.1. Place 5 µL of DAPI 125 ng/mL (DAPI II Counterstain, Abbott Molecular Inc.), in each gel and cover with a coverslip;

6.2. Keep the slides for 15 minutes, at 4ºC, in the dark;

6.3. Observe under a fluorescence microscope (Nikon Eclipse E400) at 400x magnification and rank the comets from 0 to 4 (n=100/gel) (Figure 1).

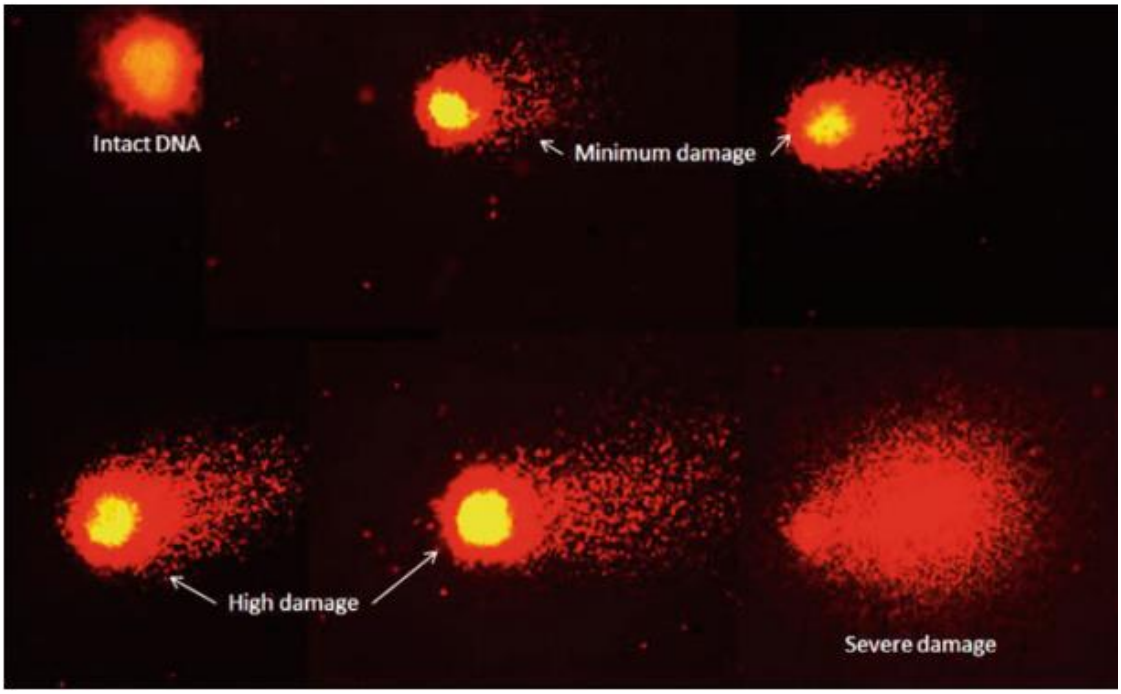


Figure 1. Comet assay. Human spermatozoa stained with ethidium bromide and observed under fluorescent microscope (400× magnifi cation). Source: Adiga & Kalthur (2016) [75].

A - Prior preparation

1. Prepare fixative and keep cold at 4ºC (3 methanol:1 acetic acid) for a centrifuge tube, adequately labelled.
2. Prepare 1x PBS for a labelled centrifuge tube. Minimum volume 5mL.
3. Prepare NaOH solution in a Coplin.
4. Prepare two Coplins with 2x SSC solution and keep them in the 37ºC bath.
5. Prepare three CoplinS with 70, 85 and 96% ethanol.
6. Prepare the humidity chamber and keep it at 37ºC

B - Protocol

1. After sample liquefaction, pipette 200uL of semen into centrifuge tube and add 1mL PBS 1x;
2. Centrifuge at 1200rpm, 10min;
3. Perform two further washes with PBS 1x (total of 3 washes);
4. Decant and carefully homogenise;
5. Add 1mL fixative (3:1 methanol/acetic acid) at 4ºC, drop by drop over vortex action. Several washes of fixative may be made until a clear pellet is obtained. In cases of samples with very low sperm concentration, adjust the amount of fixative volume. Store at -20ºC until used;
6. Pipette 10uL of the solution onto a slide, check cell density under the microscope (phase contrast) and adjust if necessary;
7. Prepare a humidity chamber and remove the probes from the cold, keeping them protected from light;
8. (Optional) Incubate the slide at -20ºC for at least 2 hours;
9. Incubate the slide in 1M NaOH solution for 2 min, RT;
10. Wash twice in a solution of 2x SSC at 37ºC, 2 min each;
11. Dehydrate in a sequence of 70, 85 and 96% ethanol baths, 2 min each;
12. Allow to dry;
13. Work in the dark;
14. Switch on the hotplate and set it to 75ºC;
15. Vortex the probe and the hybridisation solution. Spin down and homogenise each time before pipetting any volume;
16. Prepare two eppendorfs (one epp for CEP 18/X/Y and one for LSI 13/21). Add 4,5 µL of hybridisation solution to 0,5 µL of the probe. Homogenise and spin down;
17. Pipette 4,8 µL over the desired area and cover with a coverslip. Generously apply glue to the marked area;
18. The coverslips with the probes are placed under the hotplate at 75 ºC for 5min;
19. Incubate the slides at 37°C in a humidity chamber overnight;
21. In a dark environment, remove the coverslips carefully and proceed with post-hybridization washes;
22. Place the slides in 0,4x SSC with 0,3% Igepal. 72ºC, 2min;
23. Next, place the slide in 0,2x SSC for 1min;
24. Leave to dry in the dark;
25. Add DAPI, cover with a coverslip and keep at 4°C until observed.

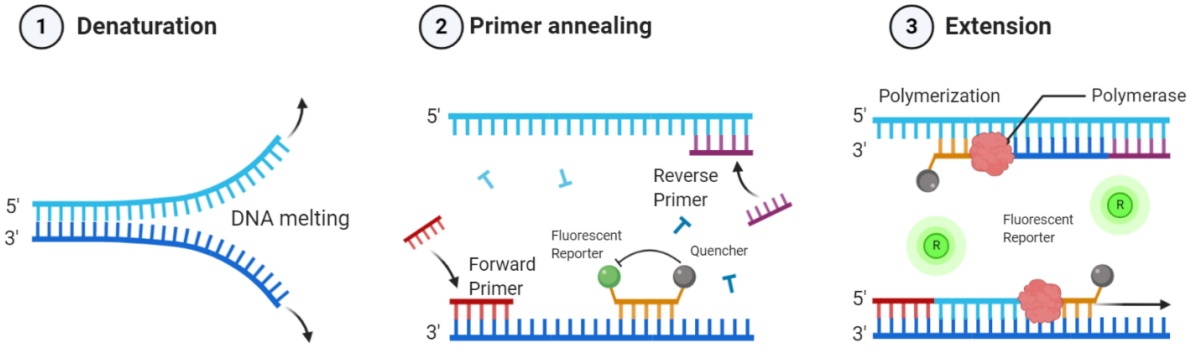


Figure 1. Illustration of probe-based qPCR for the molecular diagnosis of Y-CM. Sequence specific probes are synthesized with a fluorescent reporter dye covalently attached to the 5'end and a quencher dye to the 3' and are used in combination with primers to detect the amplification product. While the probe remains intact, the reporter dye signal is quenched. During PCR, as Taq DNA polymerase binds to and extends the primer, any probe it encounters upstream is hydrolysed and as a result, the fragment containing the reporter dye is released. The fluorescence signal can now be detected. Adapted from AAT Bioquest (2023)[106].

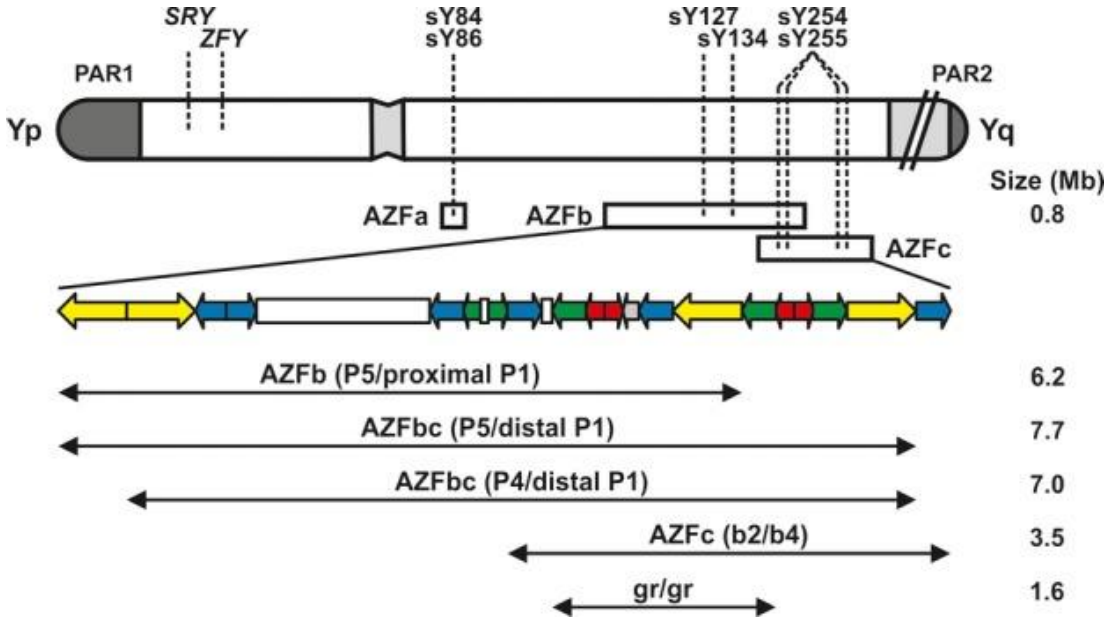


Figure 2. Schematic representation of the Y chromosome and the current microdeletion model (Repping et al., 2002). Repetitive sequences (colour coded palindromes) explain the origin of deletions in the AZFbc region by homologous recombination between identical sequences. The location of the STS primers suggested by the present guidelines is indicated by dashed lines. As four copies of the DAZ gene are normally present on the Y chromosome, the STS primers sY254, sY255 amplify four loci in AZFc. The AZFc (b2/b4) deletion is by far the most frequent type (~80%) of Y-chromosomal microdeletions found in men with severe oligo/azoospermia. Adapted from Krausz et al. (2014) [107].

Table 1. STS markers used, their respective AZF regions and qPCR test interpretation.

	STS	PCR Mix	Result Interpretation
AZFa	sY84	Mix A	AZFa deletion if no S-shaped amplification curve is present and Ct≥ 32.
	sY86	Mix B	
AZFb	sY127	Mix A	AZFb deletion if no S-shaped amplification curve is present and Ct≥ 32.
	sY134	Mix B	
AZFc	sY255	Mix A	AZFc deletion if no S-shaped amplification curve is present and Ct≥ 32.
	sY254	Mix B	
Control	*SRY*	Mix A	If an S-shaped amplification curve is present and Ct<32, results for the PCR run are valid.
	ZFX/ZFY	Mix B	

Appendix H. Semen Parameters of Population A

						Motility (%)				Sperm Count (10^6)					Morphology	
Age (y)	Class.	Abst. (d)	pH	Vol. (ml)	V (%)	IM	NP	PR	TM	C. (/ml)	TSC	TMSC	TPMSC	TNSC	% N	TZI
38	N	3	8.1	2.8	79.00	26.00	11.00	63.00	74.00	66.40	185.92	137.58	117.13	9.30	5.00	1.50
37	O	3	8.1	4.1	72.00	43.00	16.00	41.00	57.00	4.26	17.47	9.96	7.16	0.87	5.00	1.60
37	O	3	8.1	6.4						<2						
42	T	3	8.1	3.3	84.00	18.00	7.00	75.00	82.00	95.50	315.15	258.42	236.36	6.30	2.00	1.40
33	OT	3	8.1	4.5	77.00	23.00	21.00	56.00	77.00	7.93	35.69	27.48	19.98	0.36	1.00	1.80
41	N	3	8.1	2.9	86.00	30.00	15.00	55.00	70.00	48.70	141.23	98.86	77.68	9.89	7.00	1.50
36	C	4	8.3	3.2												
35	T	2	8.1	3.1	89.00	15.00	9.00	76.00	85.00	33.80	104.78	89.06	79.63	3.14	3.00	1.50
31	N	4	8.1	6.4	81.00	20.00	7.00	73.00	80.00	28.80	184.32	147.46	134.55	7.37	4.00	1.40
42	N	2	8.3	2.5	79.00	25.00	10.00	65.00	75.00	27.60	69.00	51.75	44.85	2.76	4.00	1.60
42	O	3	7.7	4.2						<2						
32	N	3	8.3	1.8	85.00	22.00	9.00	69.00	78.00	31.00	55.80	43.52	38.50	3.35	6.00	1.90
42	O	3	7.9	2.3	57.00	49.00	9.00	42.00	51.00	7.70	17.71	9.03	7.44	0.89	5.00	1.60
38	N	3	8.1	3.6	90.00	12.00	11.00	77.00	88.00	29.90	107.64	94.72	82.88	8.61	8.00	1.40
39	N	2	8.3	3.9	47.00	55.00	8.00	37.00	45.00	122.00	475.80	214.11	176.05	23.79	5.00	1.40
38	O	3	8.1	1.2	57.00	51.00	14.00	35.00	49.00	14.60	17.52	8.58	6.13	0.70	4.00	1.60
41	N	3	7.9	7.4	83.00	18.00	6.00	76.00	82.00	92.60	685.24	561.90	520.78	34.26	5.00	1.40
36	A	3	8.3	2.9	39.00	64.00	16.00	20.00	36.00	64.00	185.60	66.82	37.12	7.42	4.00	1.60
33	O	2	8.5	2.8						<2						
38	T	3	7.9	4.1	72.00	38.00	20.00	42.00	62.00	10.60	43.46	26.95	18.25	1.30	3.00	1.71
30	T	2	8.3	1.4	88.00	16.00	12.00	72.00	84.00	61.80	86.52	72.68	62.29	2.60	3.00	1.53
32	AZ	3	8.1	2.7												
41	O	3	8.3	1.2	61.00	44.00	12.00	44.00	56.00	17.98	21.58	12.08	9.49	1.29	6.00	1.50
45	O	3	8.1	1.3						<2						
34	T	3	7.7	3.2	80.00	39.00	11.00	50.00	61.00	43.50	139.20	84.91	69.60	4.18	3.00	1.40
42	O	3	8.1	4.7	66.00	48.00	17.00	35.00	52.00	6.09	28.62	14.88	10.02	1.14	4.00	1.65
43	OT	2	8.5	1.1	76.00	43.00	17.00	40.00	57.00	5.97	6.57	3.74	2.63	0.13	2.00	1.58
46	N	4	8.3	4	79.00	19.00	11.00	70.00	81.00	42.20	168.80	136.73	118.16	6.75	4.00	1.39
38	N	3	8.1	5.1	78.00	15.00	6.00	79.00	85.00	26.55	135.41	115.09	106.97	5.42	4.00	1.38
41	AZ	3	7.9	2.1												
39	N	2	7.9	4.1	70.00	39.00	7.00	54.00	61.00	12.20	50.02	30.51	27.01	2.00	4.00	1.28
38	N	3	8.1	2.8	86.00	20.00	11.00	69.00	80.00	75.90	212.52	170.02	146.64	10.63	5.00	1.23
35	T	3	8.1	2.7	68.00	32.00	18.00	50.00	68.00	61.50	166.05	112.91	83.03	4.98	3.00	1.38
35	N	2	8.3	2.1	70.00	26.00	9.00	65.00	74.00	118.50	248.85	184.15	161.75	9.95	4.00	1.28
34	T	3	8	3.8	81.00	26.00	13.00	61.00	74.00	31.00	117.80	87.17	71.86	3.53	3.00	1.25
32	AZ	3	8	2.3												
38	T	3	8	4.7	75.00	17.00	25.00	58.00	83.00	54.70	257.09	213.38	149.11	7.71	3.00	1.32
26	O	3	8.5	2.3						<2						
38	OAT	2	8	2.8	48.00	70.00	10.00	20.00	30.00	8.17	22.88	6.86	4.58	0.46	2.00	1.24
32	AZ	4	8	1.5												
30	N	3	8	4.4	82	19	8	73	81	30.7	135.08	109.41	98.61	6.75	5	1.14
42	T	2	8.5	1.7	80.00	22.00	9.00	69.00	78.00	90.30	153.51	119.74	105.92	4.61	3.00	1.25
38	O	3	8.5	3.5						<2						
28	T	3	8.5	1	80	33	14	53	67	43.63	43.63	29.23	23.12	1.31	3.00	1.39
31	T	2	8	2.3	83	34	13	53	66	65.38	150.40	99.26	79.71	4.51	3.00	1.32
40	T	3	8.5	2.8	77	26	9	65	74	23.9	66.92	49.52	43.50	2.01	3.00	1.26
44	N	3	8	3.4	79	22	10	68	78	112.8	383.50	299.13	260.78	15.34	4	1.4
35	N	3	8	2.8	85	29	9	62	71	73.3	205.20	145.69	127.22	8.21	4	1.38
39	T	2	8.5	4.1	69	33	18	49	67	10.2	41.80	28.01	20.48	0.84	2.00	1.33
47	O	3	8.5	3.2						<2						
40	T	2	8	1	75	33	22	45	67	85.8	85.80	57.49	38.61	2.57	3.00	1.34
35	OT	3	8	2.6	74	36	17	47	64	14.5	37.80	24.19	17.77	0.76	2	1.29
28	T	2	8.5	3.7	70	32	21	47	68	11.9	44.30	30.12	20.82	1.33	3.00	1.29
37	N	3	8	2.3	83	22	11	67	78	150	345.00	269.10	231.15	13.80	4	1.23
29	OT	3	8	4.8	58	72	20	8	28	5.89	28.30	7.92	2.26	0.85	3	1.22
38	T	2	8.5	2.7	86	14	5	81	86	25.8	69.70	59.94	56.46	2.09	3.00	1.32

Abst., abstinence period; Class., WHO semen classification; Vol, volume; V, vitality, IM, immotile; NP, nonprogressive; PR, progressive; TM, total motility; C., Concentration; TSC., total sperm count; TMSC, total motile sperm concentration; TPMSC, total progressive motile sperm concentration; TNSC, total normal sperm count; NT, normal/typical sperm forms; TZI, teratozoospermia index; d, days; y, years; <2, less than 2 million sperm cells/ml; A, asthenozoospermia; AT, asthenoteratozoospermia; AZ, azoospermia; C, criptozoospermia; N, normozoospermia; O, oligozoospermia; AO, oligoasthenozoospermia; OAT, oligoasthenoteratozoospermia; OT, oligoteratozoospermia; T, teratozoospermia.

Appendix I. Semen Parameters of Population B

						Motility				Sperm Count (10^6 Sperm cells)					Morphology	
Age (y)	Class.	Abst. (d)	pH	Vol (mL)	V (%)	IM	NP	PR	TM	Conc. (/mL)	TSC	TMSC	TPMSC	TNSC	%N	TZI
22	N	2	8.00	0.80	72.00	28.00	10.00	62.00	72.00	192.30	153.80	110.74	95.36	7.69	5.00	1.44
24	N	3	7.90	1.10	70.00	36.00	10.00	54.00	64.00	58.75	64.00	40.96	34.56	3.20	5.00	1.50
21	N	3	7.93	2.00	75.00	26.00	12.00	62.00	74.00	111.50	223.00	165.02	138.26	8.92	4.00	1.54
40	N	3	7.90	2.40	89.00	16.00	6.00	78.00	84.00	23.00	55.20	46.37	43.06	3.86	7.00	1.60
22	N	2	8.00	2.40	79.00	25.00	9.00	66.00	75.00	182.00	436.80	327.60	288.29	21.84	5.00	1.49
22	N	2	8.00	2.50	92.00	26.00	3.00	71.00	74.00	49.60	124.00	91.76	88.04	6.20	5.00	1.42
40	N	2	8.00	3.00	90.00	18.00	6.00	76.00	82.00	46.00	138.00	113.16	104.88	5.52	4.00	1.50
28	N	5	8.50	3.00	85.00	33.00	12.00	55.00	67.00	88.13	264.39	177.14	145.41	21.15	8.00	1.54
30	N	4	8.00	10.10	85.00	17.00	15.00	68.00	83.00	5.21	52.62	43.67	35.78	5.26	10.00	1.52
24	OS	3	8.00	1.20	81.00	34.00	13.00	53.00	66.00	5.53	6.64	4.38	3.52	0.27	4.00	1.53
22	T	2	8.00	2.00	79.00	47.00	7.00	46.00	53.00	55.90	111.80	59.25	51.43	2.24	2.00	1.46
22	T	2	8.50	3.50	85.00	15.00	10.00	75.00	85.00	55.88	195.58	166.24	146.69	3.91	2.00	1.68
18	T	3	8.00	4.60	83.00	26.00	6.00	68.00	74.00	44.00	202.40	149.78	137.63	4.05	2.00	1.40

Abst., abstinence period; Class., WHO semen classification; Vol, volume; V, vitality, IM, immotile; NP, nonprogressive; PR, progressive; TM, total motility; C., Concentration; TSC., total sperm count; TMSC, total motile sperm concentration; TPMSC, total progressive motile sperm concentration; TNSC, total normal sperm count; NT, normal/typical sperm forms; TZI, teratozoospermia index; d, days; y, years; A, asthenozoospermia; AT, asthenoteratozoospermia; AZ, azoospermia; C, criptozoospermia; N, normozoospermia; O, oligozoospermia; AO, oligoasthenozoospermia; OAT, oligoasthenoteratozoospermia; OT, oligoteratozoospermia; T, teratozoospermia.

A. Direct Swim-up

1. Allow semen sample to liquefy completely in a 37°C incubator;
2. Transfer the semen sample into 15ml sterile round bottom tubes using a sterile serological pipette;
3. Stratify, above the semen sample, 1 ml of culture medium (Sperm Preparation Medium, Origio, Denmark)
4. Incubate the tubes at a 45° angle for 1 hour in vertical rack in a 37°C incubator;
5. Aspirate the supernatant and transfer into an empty tube;
6. Assess motility, concentration, and morphology

B. Simple Wash

1. Allow semen sample to liquefy completely in a 37°C incubator;
2. Transfer the semen sample into 15ml sterile conical bottom tubes using a sterile serological pipette;
3. Add SPM (Origio, Denmark) 1:1 and homogenise;
4. Centrifuge at 300g for the desired time;
5. Discard the supernatant.
6. Add 1ml SPM (Origio, Denmark) and gently resuspend the pellet;
7. Centrifuge at 300g for the desired time;
8. Discard the supernatant and resuspend the pellet in the desired SPM volume;
9. Assess motility and concentration.