Internship Report in Assisted Human Reproduction: The Andrology and Embryology Laboratory

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"*Put love out into the world*"

"*There was finally enough air within her for a fire to ignite*"

Resumo

A infertilidade é considerada na atualidade um problema de saúde pública global, o que constituiu um impulsionador para desenvolvimentos na área da medicina reprodutiva, permitindo abordar esta questão através de diversas técnicas de reprodução assistida, tais como a inseminação intrauterina, a fertilização *in vitro* e a injeção intracitoplasmática de espermatozoides. Estes procedimentos permitem transpor as barreiras à conceção presentes em casais inférteis, sendo que a escolha do tratamento correto a adotar é dependente da obtenção de um diagnóstico, visando potenciar a ocorrência de gravidez. Os laboratórios de andrologia e embriologia são os locais onde estes essenciais e complexos procedimentos decorrem, preparados e executados por embriologistas que procuram atingir condições, processos e resultados ideais, ao tomar decisões de forma a otimizar a ocorrência de gravidez e parto por parte dos pacientes.

Neste relatório, foi tido como objetivo escrever um manual de laboratório de procriação medicamente assistida, através de uma visão global da literatura incluindo conceitos e procedimentos fundamentais ao trabalho de laboratório nesta área. Os campos da embriologia e andrologia estão em inovação e evolução contínua, com inúmeras técnicas em investigação e desenvolvimento, sendo que este relatório se foca principalmente em técnicas com as quais tive contacto durante o estágio.

Adicionalmente, foi realizada uma análise estatística retrospetiva com dados clínicos, com o objetivo de compreender quais parâmetros do ciclo e/ou dos pacientes têm influência significativa na probabilidade de ocorrência de gravidez após o primeiro ciclo de inseminação intrauterina nos casais inférteis da presente coorte. Através de modelos de regressão logística binomial, os hábitos de tabagismo femininos e a causa de infertilidade foram obtidos como fatores relevantes para a ocorrência de gravidez, expondo a importância da sua consideração para a individualização dos ciclos de tratamento com recorrência a inseminação intrauterina, de forma a otimizar as taxas de conceção.

Palavras-chave: Infertilidade, Procriação medicamente assistida, Inseminação intrauterina, Fertilização *in vitro*, Injeção intracitoplasmática de espermatozoides, Gâmetas, Embriões, Gravidez.

Abstract

Infertility has become a global healthcare concern over recent decades, prompting developments in the reproductive medicine field to address this issue through various assisted reproductive technologies, such as intrauterine insemination, *in vitro* fertilisation, and intracytoplasmic sperm injection. These procedures can overcome the barriers to conception faced by infertile couples, with the choice of treatment contingent upon an accurate diagnosis to ensure that the chance of pregnancy is optimised. The andrology and embryology laboratories are the settings in which these intricate and crucial procedures are prepared and carried out by embryologists, striving for ideal conditions, processes and results, while making decisions that pursue the achievement of pregnancy and live birth by the patients.

In this report, the aim was to write a comprehensive assisted human reproduction laboratory manual, through a literature overview including fundamental concepts and procedures that are essential for laboratory work in this specialty. The fields of andrology and embryology continually evolve with constant innovation, with many techniques still ongoing research and development, but this report primarily focuses on techniques and procedures I encountered during my internship.

Additionally, a retrospective statistical analysis with clinical data was conducted, with the purpose of unveiling which patient and/or cycle parameters exert a significant influence in the odds of achieving clinical pregnancy following the first intrauterine insemination cycle in infertile couples. Through binomial logistic modelling, female smoking habits and infertility diagnosis were the parameters that were shown to be relevant for clinical pregnancy, and consequently, are factors that should be considered for individual optimisation of insemination cycles.

Keywords: Infertility, Assisted Reproductive Technology, Intrauterine insemination, *In vitro* fertilisation, Intracytoplasmic sperm injection, Gametes, Embryos, Clinical pregnancy.

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List of Abbreviations

AMA	Advanced Maternal Age
AOA	Artificial Oocyte Activation
APA	Advanced Paternal Age
ART	Assisted Reproductive Technology
BMI	Body Mass Index
CI	Confidence Interval
COC	Cumulus-Oocyte Complexes
CP	Clinical Pregnancy
cTESE	Conventional Testicular Sperm Extraction
DGC	Density Gradient Centrifugation
EQ	Embryo Quality
eSET	Elective Single Embryo Transfer
ET	Embryo Transfer
FR	Fertilisation Rate
GV	Germinal Vesicle
hCG	Human chorionic gonadotropin
hpi	Hours Post-Insemination or Injection
HSM	*Hospital de Santa Maria*
ICM	Inner Cell Mass
ICSI	Intracytoplasmic Sperm Injection
IR	Implantation Rate
IUI	Intrauterine Insemination
IVF	*In Vitro* Fertilisation
LBR	Live Birth Rate
MAR	Medically Assisted Reproduction
MESA	Microsurgical Epididymal Sperm Aspiration
MI	Metaphase I
MII	Metaphase II
MSS	Microfluidic Sperm Sorting
mTESE	Micro Testicular Sperm Extraction
NOA	Non-Obstructive Azoospermia
NSS	Non-Stage Specific
OA	Obstructive Azoospermia
OPU	Ovum Pick-Up

OR	Odds Ratio
PCOS	Polycystic Ovary Syndrome
PESA	Percutaneous Epididymal Sperm Aspiration
PN	Pronuclei
POI	Premature Ovarian Insufficiency
PR	Pregnancy Rate
PVP	Polyvinylpyrrolidone
ROS	Reactive Oxygen Species
SCO	Sertoli Cell Only Syndrome
SCSA	Sperm Chromatin Structure Assay
SD	Standard Deviation
SDF	Sperm DNA Fragmentation
sER	Smooth Endoplasmic Reticulum
SS	Stage Specific
STD	Sexually Transmitted Disease
SU	Swim Up
TE	Trophectoderm
TESA	Testicular Sperm Aspiration
TESE	Testicular Sperm Extraction
TFF	Total Fertilisation Failure
UMR	*Unidade de Medicina da Reprodução*
WHO	World Health Organization

Chapter 1 – Assisted Human Reproduction

1. Infertility

1.1 Definition

Infertility is characterized by the World Health Organisation (WHO) as a disease of the male or female reproductive system, that is defined by the failure to establish a clinical pregnancy after at least 12 months of regular, unprotected sexual intercourse[1]. For women over 35 years old, this definition is slightly adapted to at least 6 months of regular, unprotected sexual intercourse, so that fertility testing and treatment may be anticipated[2,3]. It is of great importance to denote that this definition does not cover every individual or couple who is diagnosed with infertility and needs to resort to fertility treatments to fulfil their reproductive wishes. The International Glossary on Infertility and Fertility Care published in 2017 presents infertility as a disease characterized by the WHO definition, or additionally characterized by the incapacity of reproduction as an individual person or with a partner[4]. This definition is more inclusive and aims to include same-sex couples, individual persons, and opposite-sex couples with any impairment to reproduction.

Infertility is considered a global healthcare concern, having impact on the quality of life, social aspects, and psychological conditions as well as physical health of affected individuals[5,6]. Worldwide, it affects 15-20% of couples within the reproductive age[7], with an estimated number of 48.5 million infertile couples in 2010[8]. According to the latest WHO report, with data from 1990 to 2021, approximately 1 in 6 people have experienced infertility in their lifetimes[9].

1.1.2 Types of Infertility

Infertility can be diagnosed as primary, in cases where there has never been a clinical pregnancy, or secondary, when there has already been at least one clinical pregnancy in the individual's/couples' reproductive history prior to the diagnosis[1,4].

1.2 Causes of infertility

Infertility is a highly complex disease, that can be caused or influenced by a wide array of female or male factors, or even a combination of both[10]. Additionally, in

10-30% of cases it is not possible to pinpoint the exact causes for infertility, in which case, the infertility is taken as unexplained[10,11].

1.2.1 Female factor

The aetiology of the female factor for infertility can be classified into four main categories: ovarian factor, tubo-peritoneal factor, uterine factor and cervical factor. Within these, the most common causes for female infertility fall into the ovarian and tubo-peritoneal factors[12].

1.2.1.1 Ovarian factor

Ovarian factor infertility is generally related to ovulatory dysfunction[12]. This infertility cause is identified in around 25% of all infertile couples, and it can be caused by a variety of underlying factors[13].

The most frequent causes of ovulatory dysfunction include polycystic ovary syndrome (PCOS), other endocrine disorders or ovarian failure[2,6,13].

PCOS is the most prevalent hormonal disorder in women of reproductive age, affecting 5-10% of women, and being the primary cause of anovulatory infertility, accounting for up to 70% of cases[14,15].

Premature ovarian insufficiency (POI) is one type of ovarian failure defined by WHO[14], characterised by the loss of ovarian function prior to the age of 40 years old, including women with premature menopause, and its prevalence is about 1% in the general population[4,16]. The most common genetic cause for POI is Turner syndrome, characterized by X chromosome-related abnormalities[17,18].

Other types of ovarian factor include genetic, autoimmune, infection or iatrogenic-related causes[14,19]. Genetic-related abnormalities derived from meiosis errors may compromise oocyte's viability and competence, which can be associated with fertilisation or implantation failure and miscarriages[19].

1.2.1.2 Tubo-peritoneal factor

Tubal factor encompasses the impairment of the Fallopian duct function, that can be due to partial or total occlusion or functional alteration of one or both tubes[4,6].

It can have inflammatory disease-related origin, mainly related to pelvic infections, arising from untreated sexually transmitted diseases (STDs)[6]. Tubal

diseases are responsible for 25-35% of female factor infertility, one of the most frequent aetiologies[2]. Other causes for tubal factor infertility may be congenital malformations, development of adhesions after pelvic surgery and disorders of ciliary function[4,6].

Peritoneal factor is mainly related to endometriosis and pelvic adhesions[2]. Endometriosis is a chronic pelvic inflammatory disease that is characterized by the presence of endometrium-like epithelium outside the uterine cavity, pelvic adhesions, as well as endocrine and immunological disturbances[4,20]. It affects about 5-10% of fertile-age women[6], and is a contributing cause in about 25-40% of infertility cases[21].

1.2.1.3 Uterine factor

Uterine factor can be defined by a lack of uterus, or the presence of a non-functional uterus, and it is responsible for 2-17% of female infertility cases[22]. It is mainly associated with congenital or acquired uterine abnormalities, and the most common congenital abnormality, present in 35% of cases, are uterine septa[6,23,24]. Acquired uterine abnormalities range from polyps, fibroids, myomas and synechiae[12,22,23].

These alterations usually have both anatomical and functional adverse consequences[2], affecting endometrial receptivity and implantation or preventing pregnancy development, thus reducing fecundability and increasing the chances of spontaneous abortion and preterm delivery[12,24].

1.2.1.4 Cervical factor

In order to achieve fertilisation of the oocyte, the spermatozoa must pass through the cervix, from the vagina to the uterus. Cervical factor infertility arises when this step is not possible or hindered[12]. It may be caused by damage to the cervix, infections, congenital malformations, or alterations to the physicochemical properties of the cervical mucus, converting it from a sperm transport-favourable nature to a non-receptive one[12]. In most cases, this factor is not the sole cause of infertility[2,13].

1.2.2 Male factor

Male infertility has a multifactorial nature, that can be related to hormonal imbalances, anatomical defects, STDs, genetic factors, extrinsic agents, sexual dysfunction and other risk factors[4,25–27].

It has been reported that chromosomal defects have a great relevance in male infertility, being identified in 15% of infertile men[25,28,29]. Although around 40-50% of male infertility cases remain unexplained[30], it is thought that many of these cases would be explained by unknown molecular or genetic abnormalities[29,31]. Sperm DNA fragmentation (SDF) caused by elevated levels of reactive oxygen species (ROS) is given as one of the significant possible causes in these cases[10,32,33].

Besides the heterogeneity, the aetiologies for male infertility can be categorized into pre-testicular, testicular and post-testicular[34]. Additionally, pre-testicular and testicular factors can be grouped together into non-obstructive causes for male infertility, that are more common than obstructive causes (post-testicular factors)[35]. A correct diagnosis is crucial when it comes to male factor infertility, because the identification of the cause for infertility is highly associated with successful treatment[28,34].

1.2.2.1 Pre-testicular factor

The pre-testicular causes for male infertility origin from outside the reproductive system, and fall mainly on endocrine imbalances, having an impact on the hormonal regulation of spermatogenesis[25]. A defect on any step of the axis that regulates testosterone production on the testis has the potential to lead to this dysregulation, also called secondary testicular failure[25,36]. Consequently, the impact falls on a defective spermatogenesis[28] or even non-obstructive azoospermia (NOA), characterized by an absence of sperm in the ejaculate[4]. Only 1-2% of infertile men are affected by pre-testicular factor infertility[37].

Pre-testicular factor can rise from the action of extrinsic agents, such as lifestyle factors and damage to the hypothalamus[28,32]. Alternatively, it can also be caused by genetic disorders, such as Kallman syndrome, or by oncologic diseases, such as pituitary tumours[34–36].

1.2.2.2 Testicular factor

The testicular causes for male infertility are the ones that have a direct impact on the testicles, and consequently, on male reproductive function[36]. In these cases, spermatogenesis is likely to be affected, which can lead to altered sperm parameters or NOA[38]. This primary testicular failure is the most frequent cause of male infertility and NOA[10,32].

Testicular factor may arise from congenital causes, usually associated with chromosomal disorders such as Y chromosome microdeletions in the *AZF* region or Klinefelter syndrome[23,36].

Klinefelter syndrome is characterized by the XXY genotype, and it is associated with NOA[32,35]. It is the most common chromosomal disorder associated with male infertility, accounting for approximately 8% of infertile men[39], and reports suggest that in up to 50% of affected men, it is possible to retrieve sperm to be used in ART treatments[38,40].

Concerning the *AZF* region, it has been reported that microdeletions in this region may be associated with severe spermatogenesis impairment[34]. These microdeletions have been found in only <1% of men with normal semen parameters[41], but in up to 13% of azoospermic men[42]. *AZFc* region microdeletions are more prevalent, accounting to 65-70% of cases, and are associated with a less severe impact on fertility[10,30,32,41]. *AZFa* region microdeletions are the rarest, with a prevalence of 5%, and can be responsible for Sertoli cell only syndrome (SCO), a cause of NOA[30,34,43].

A congenital non-genetic disorder that also affects males and causes testicular factor infertility is a history of cryptorchidism[20,23], with a reported 2-5% incidence at birth[30]. It is defined by the incomplete descent of testis into the scrotum[4], which can lead to impaired spermatogenesis due to the elevated temperature[44]. In the infertile population, about 10% of men have history of cryptorchidism[45].

Conversely, infertility by testicular factor can also by acquired, when external factors such as injury, disease, infection, varicocele, chemo- or radiotherapy occur, or derived from lifestyle and/or environmental factors[20,28,34,35].

Varicocele is characterized by an enlargement in the spermatic veins, that leads to poor semen quality in all conventional parameters, plus DNA fragmentation[4,46]. It is thought to affect spermatogenesis in a multifactorial manner, but mainly through scrotal heating and consequent increase in the formation of ROS[32,35]. It has been reported to be present in about 25% of men with abnormal sperm parameters[47].

1.2.2.3 Post-testicular factor

The post-testicular causes for male infertility refer mainly to the ones that cause obstruction in the ducts of the male reproductive tract, preventing spermatozoa and semen from being transported during ejaculation[34,35]. Therefore, in this case, there is

no impairment of sperm production in the testicles[35]. It can lead to a low concentration of sperm in the ejaculate, or even obstructive azoospermia (OA)[28]. In the majority of OA cases, there is a high potential for successful sperm retrieval for use in ART treatments[48].

The obstruction can be caused by various acquired reasons, such as trauma, infection and surgery, or congenital abnormalities in the reproductive tract[34,35,48]. A congenital bilateral absence of the vas deferens is the cause of 6% of cases of OA[49], and is present in 98% of patients diagnosed with the genetic disease cystic fibrosis[23,50].

Other causes of post-testicular male infertility are retrograde ejaculation, in which instead of being directed to the urethra, the ejaculate is expelled to the bladder, and erectile or ejaculatory dysfunction, both of which do not allow the expulsion of the ejaculate[34,35,51]. Furthermore, post-testicular infertility can be related to disorders of sperm function, such as immunological infertility (presence of anti-sperm antibodies) and defects in spermatozoa maturation in the epididymis[34,35,48].

1.2.3 Unexplained infertility

Unexplained infertility is considered a diagnosis of exclusion where standard fertility testing is used to assess potential causes of infertility, and no apparent biological cause explains the inability to conceive, that is present in 15-30% of infertile couples[6,12,23]. Usually, it requires the exclusion of anatomical and functional male and female causes, as well as adequate coital frequency[4].

In these cases, it is thought that the diminished fecundity may be related to several subtle female and/or male factors combined[12]. Unexplained infertility has been linked to a wide range of causes, from high SDF and presence of anti-sperm antibodies in men[33], to poor oocyte quality, low ovarian reserve and undiagnosed tubal disease or endometriosis in women[52–54]. It has been suggested that unexplained infertility might be intertwined with a heterogeneous group of cellular abnormalities, not detected by the standard fertility tests, that do not cover every step in the reproductive process, missing, per example, implantation and the interaction between sperm and oocyte[33,52,53,55].

The female age and the duration of infertility are the two most critical prognostic markers in couples with unexplained infertility[23,56]. Despite the inability to identify the cause for infertility, couples with this diagnosis have comparable results in reproductive outcomes after ART treatments, compared with other infertility aetiologies, such as

male factor, PCOS and tubal factor[52,54,57]. Even between ART treatments, there seems to be no significant difference in probability of success of intrauterine insemination (IUI) compared with *in vitro* fertilisation (IVF) with ovarian stimulation in couples with unexplained infertility, in which the female is aged between 18 and 38 years[56,58,59]. However, in women aged between 38 and 42 years, a significantly higher pregnancy rate and lower time to pregnancy has been reported with IVF rather than IUI[56,60]. Regarding chances of spontaneous pregnancy, unexplained infertility has been shown to be associated with an increased probability when compared to other infertility aetiologies[23,61].

1.3 Risk factors

Apart from pathological causes for infertility, other biological causes and external factors have a significant impact on fertility.

1.3.1 Advanced age

The main issue with advanced age and fertility is the incompatible nature of today's societal trends and the physiological age for reproduction, especially for women[62]. The continuing education, career goals, availability of safe and effective contraception and access to reproductive technologies in Western societies are indicated as possible reasons for the postponing of mother- and fatherhood to later stages of reproductive life[20,62,63].

An increase in women with first births in their late 20s, 30s and 40s has been reported, as well as a decline of that statistic in women in their early 20s[64]. In 2021, the mean age for maternal age at first birth in the United States was 27.3 years old, and the mean maternal age in the United Kingdom and Wales was reported to be 30.9 years old, both representing the highest national records[64,65]. In 2010, the majority of births occurred among women over 30 years old in Spain, France and Czech Republic[66].

Female age is a key factor for infertility, being one of the central infertility risk factors overall[6,67]. The declining of the female biological capacity to reproduce starts as soon as 25-30 years old[20], but is observed especially after 35 years of age[63,68].

Therefore, the advanced age question is relevant especially for women, because it poises as a physiological decline in fertility, not only due to the depletion of the ovarian reserve, but also because of the impact on the quality of oocytes, mainly

related to chromosomal aneuploidy, leading to a higher incidence of, for instance, Trisomy 21 in the offspring of women over 35 years old[6,68–70]. Hence, the negative impacts and risks on reproductive outcomes in older women are verified overall, both on natural conception and ART procedures[68]. Additionally, advanced maternal age (AMA) is also associated with higher probability for gestation and delivery complications, including miscarriage[62].

As for advanced paternal age (APA), its role on infertility is less characterized, but there has been evidence that points to a less profound but significant impact on different aspects of fecundity[66,71]. In 2021, the mean age of fathers in the United Kingdom and Wales was 33.7 years old, the highest ever recorded[65].

In men over 40 years old, the main remark is an impaired semen quality, with a larger effect on the decrease of progressive motility and increase of SDF[71–73]. Particularly, the occurrence of DNA mutations during the mitotic replications in the germ line are reflected on the offspring's development of genetic disorders[63,72,74]. There is also data that points to a greater independent risk of congenital anomalies, mental disorders such as autism spectrum disorder and schizophrenia, as well as spontaneous miscarriage as paternal age increases[71,74,75].

Even though the resort to ART provides better reproductive outcomes for couples with AMA or APA, it may not be possible to overcome the underlying loss of fecundity[66]. In the case of women with decreased ovarian reserve, using donor eggs may be the only option available for viable ART treatment[62].

In a couple where AMA, APA or both are present, ART outcomes ought to be affected, although it is debated in which rates and treatments that impact is seen. The reason for this is because it is difficult to study the impact of each of these factors separately, but generally, fertilisation, implantation, pregnancy, live birth rates and embryo quality are decreased[76–79].

1.3.2 Environmental factors

Anthropogenic stressors, such as pollutants, toxins and radiation stand out as environmental risks for fertility, which may be associated with a higher incidence of infertility due to their possible effect on male and female gametes[80].

Environmental pollutants, specifically air and chemical pollution, play a significant role in the health of the general population, especially male and female reproductive function, by directly or indirectly affecting gonadal steroidogenesis and

gametogenesis through endocrine disruption, generation of oxidative stress and/or DNA alterations[80–82]. Both the quantity and quality of gametes can be affected, as well as the outcomes of IVF treatments[67,80,81].

Specifically for male fertility, it has been shown that distinct forms of air pollution cause an overall decrease of sperm quality, by worsening all sperm parameters, even DNA damage[83,84], with the risk of impairing testicular function[26,80].

Even though the study of the pollutant's effect on female fertility is less common, it can be said that the impact on women's gametes is worse than on men's, due to the finite pool of germ cells present in the ovaries[67]. The most agreed on consequences of air pollution on female fertility range from a depletion of primordial and antral ovarian follicle numbers, causing the decrease of ovarian reserve, a lower fertility rate, higher implantation failure, and higher miscarriage rates, lowering fecundability as a whole[67,80,81].

Additionally, the increase in worldwide temperature can also have a negative influence on the majority of sperm parameters[82,85], and there has been some evidence pointing to an impact on oocyte maturation, and consequently, on embryo development in animals[86,87].

1.3.3 Lifestyle factors

The impact of lifestyle has been increasingly considered as a significant factor for infertility[55]. Harmful lifestyles that have been demonstrated to pose as risks to fertility and reproductive health encompass having an excessive consumption of alcohol, tobacco or drugs, obesity and excessive exercise[28,88]. Moreover, occupational risk to fertility is present in professions that entail prolonged periods of sitting or exposure to high temperatures or chemical agents[26,82,89]. The risks related to some of these factors are something that can possibly be prevented or modified, to improve not only reproductive health, but also ART outcomes[67,82,90].

Both smoking and drinking have been demonstrated to cause sperm abnormalities, affecting especially sperm count and morphology in a dose-dependent manner in fertile and infertile men[91–93]. It has been reported that these negative consequences were no longer observed in men considered former smokers, as well as in men after 10-12 weeks of no alcohol drinking[93,94]. In women, smoking and heavy consumption of alcohol are associated with poor oocyte quality, follicular depletion and consequently, early menopause[90,95]. Additionally, alcohol or tobacco consumption

during pregnancy causes prenatal exposure to these substances, which has been reported to influence the male offspring's sperm quality and female offspring's germ cell quantity[96,97].

Moreover, both obesity and psychological stress are associated with worse semen quality mainly due to the decreased levels of testosterone[98–100]. In regard to the impact of changing habits, weight loss has been associated with an increase in sperm morphology and decrease in DNA damage in men[101]. The impact of obesity in female fertility falls on higher probability of anovulation and decreased endometrial receptivity and oocyte quality, which reduce ART cycles' success[102,103]. Similarly to men, weight loss also contributes to the improvement of reproductive function[102–104].

The occupational risk related to increased scrotal temperature, that is very widespread in a range of professions, either sedentary or that entail heavy labour, also may lead to an impaired male fertility, because spermatogenesis is highly sensitive to temperature alterations[28,82].

1.4 The decline in male fertility

In 1992, the first report on the decreasing quality of sperm brought forward the discussion about the overall decline of sperm parameters[27,105]. Carlsen et al. reported that the mean sperm concentration significantly decreased between 1938 and 1991, going from 113 x 10^6/mL to 66 x 10^6/mL in men without a history of infertility[105].

More recently, a meta-analysis of temporal trends in sperm counts has reported a global and continuing decline in sperm concentration and total sperm count, from 101.2 x 10^6/mL and 335.7 x 10^6 in 1973 to 49.0 x 10^6/mL and 126.6 x 10^6 in 2018, in unselected men from across all continents. The highest decline was found in the group of unselected men from North America, Europe, and Australia[106]. This decline was found to be particularly substantial in the 21st century, as the percent decline in sperm concentration per year was 1.16% post-1972, but 2.64% post-2000, and is already considered a public health concern[32,106]. Sperm quality does not only have reproductive implications, but it has been shown to be an accurate biomarker for general male health[27], with associations to long-term morbidity and risk of adverse health outcomes[107–109]. Specifically, it has been reported that men with sperm concentrations bellow 15 x 10^6/mL have a higher probability to be hospitalized earlier than men with sperm concentrations above 40 x 10^6/mL[109], and that azoospermic men are at a higher risk of death, compared to men with oligospermia[110].

Although the decline in sperm quality remains a controversial topic, mainly due to the heterogeneity of uncontrollable factors that affect sperm quality and its analysis, the declining sperm quality trend is in accordance with the growing relevant impact of lifestyle and environmental factors in reproductive health[27].

2. Male gametes

2.1 Collection

The primary recommendation for male sample collection is masturbation within the clinic, due to being a simple and non-invasive method[111,112]. Instructions must be given to each patient prior to collection, to guarantee a correct collection and a non-contaminated semen sample[112,113]. The collection is made into a wide necked, sterile, disposable, non-toxic for spermatozoa, room temperature, and identified container[112,113]. Additionally, is it important for the patient to file a questionnaire regarding the period of abstinence prior to the collection, and whether the collected sample corresponds to the complete ejaculate[112,114,115]. It is essential for identity to be confirmed in the moment, usually through the patient's ID[116].

It is recommended that sexual abstinence should be of two to seven days prior to semen analysis according to WHO, or of two to five days, according to the American Society for Reproductive Medicine[112,117]. It is important to be aware of variations in the abstinence period, as it may affect the quality of the sample, although these associations are not clearly defined[118]. When abstinence is of >7 days, sperm motility may be affected, and when it is of <2 days, semen volume and sperm concentration may be reduced, while sperm motility may improve and SDF may decrease[119–121]. The ejaculate is composed of two different fractions, and given that the first is a sperm-rich fraction, with higher concentration and motility[122], it is also important to know if the collection was complete, especially because in cases where the first fraction was lost, it may lead to false results[112,123].

It is important that the collection is made in the proximity of the andrology laboratory, so that within 30-60 minutes, the sample is being processed[111–114]. However, when that is not possible for the patient, the sample can be collected outside the clinic[112,113]. In those cases, it is essential that the sample is delivered to the laboratory within 30-50 minutes after collection, and that room temperature is maintained during the transport, to maintain sperm viability[115,116].

2.1.1 Biopsy

In some cases, an alternative mode of sperm collection is necessary, in which the male gametes are surgically retrieved directly from the testis or the epididymis, ideally with minimal trauma[4,40], in order to obtain motile sperm to be used in ART

treatments, specifically intracytoplasmic sperm injection (ICSI)[40,124]. This is the option for cases in which there has already been two sperm analysis where no spermatozoa were found in the ejaculate or in cases of retrograde ejaculation and other sexual dysfunctions[125,126]. For that effect, sperm retrieval can be performed on the day of oocyte retrieval or the day prior[115]. If possible, it is recommended that a part of the recovered spermatozoa are cryopreserved, to be used in subsequent treatments without the need for an additional surgical procedure[40,124,127].

The procedures can be performed uni- or bilaterally on the testis through testicular sperm aspiration (TESA) or testicular sperm extraction (TESE), or on the epididymis through microsurgical epididymal sperm aspiration (MESA) or percutaneous epididymal sperm aspiration (PESA)[115,126]. TESA is performed by testis puncture and aspiration, and TESE is an open procedure, in which portions of testicular tissue are excised[40,115]. PESA relies on the aspiration of epididymal fluid through puncture, while MESA is based on aspirating the fluid after opening the epididymal tubules[115]. Both PESA and TESA are adequate for patients with OA[112,124].

PESA and TESA are simple and minimally invasive procedures, but the blind aspiration leads to a lower sperm retrieval[40,128]. MESA usually has higher yields in comparison to PESA, although it is a more complex procedure[124,128].

Additionally, there is also the option of micro-TESE (mTESE), especially in cases of NOA[48,129], in which the seminiferous tubules are inspected with a surgical microscope and only the ones showing signs of harbouring higher quantities of sperm cells, rare in NOA cases, are excised[40,130]. Compared to conventional TESE (cTESE), mTESE decreases the quantity of tissue that is removed while increasing recovery rates[124,130], and is associated with less complications and testicular damage[126,128,129]. If sperm retrieval cannot be achieved even through these methods, the remaining treatment option is resorting to donor sperm[40,44].

The processing of samples obtained from testicular biopsies, namely TESE (cTESE and mTESE) and TESA, includes the mechanical shredding of the tissue in washing medium, to dislodge sperm from the seminiferous tubules, followed by a search for motile spermatozoa with an inverted microscope[115,124,127]. However, these samples are usually rich in non-sperm cells, and it can be difficult to identify spermatozoa[112,115]. If the sample is recovered from the epididymis, the recovered fluid mixed with buffered medium is directly inspected under the inverted microscope[115,124]. It is more likely to find a high quantity of mature and motile sperm cells in samples derived from epididymal aspiration than from testicular retrieval, given that sperm

maturation and storage occurs in the epididymis[40,115]. The technique of further processing of the sperm is dependent on the quality of the sample, and may rely on density gradients or simple washes[112,124].

It is expected that in cases of OA, sperm can be obtained in all cases with surgical retrieval, while that is restricted or impossible in cases of NOA[40]. For testicular retrieval, the success is dependent on the sperm production, as it is common to find focal spermatogenesis-active areas in the testis[40,126].

2.2 Semen analysis

The fundamental approach to evaluate male infertility has remained performing a semen analysis[113,123]. Besides the semen processing on the day of the oocyte retrieval, semen analysis is performed as a diagnostic tool[5,115], given that the analysed parameters reflect the male reproductive system's functionality[112,123]. In a diagnostic standpoint, it ascertains the presence of a male factor, and its severity[117,123].

Given the possible high variability of ejaculate characteristics in between collections of the same subject[120,131], it may be necessary to perform a second semen analysis to increase the validity of the results[113,114], especially in cases of poor semen quality[120,123]. In cases where the results are normal, a single analysis might be sufficient[112,132].

It is of high interest to standardize semen processing and examination, in order to guarantee quality care for patients across ART laboratories, which is the purpose of the WHO Laboratory Manual for the Examination and Processing of Human Semen[112]. As such, semen analysis is conducted in accordance with the WHO Manual, and encompasses macroscopic and microscopic parameters (**Table 1**)[111,123].

Table 1: Sperm parameters that are analysed on a standard semen analysis[112,125].

Sperm analysis parameters		
Macroscopic	**Microscopic**	
Liquefaction	Motility	Vitality
Volume	Concentration	Presence of non-sperm cells
Viscosity	Morphology	
Appearance	Agglutination	
pH		

The semen should be swirled into a homogeneous sample before analysis, handled in a laminar flow hood, and the used media must be appropriate for sperm in IVF, equilibrated and heated at 37ºC[112–114].

2.2.1 Macroscopic

The macroscopic parameters for semen analysis are not as exact as the microscopic parameters, but clinical significance can still be derived from their values[112].

2.2.1.1 Liquefaction

The analysis should start 30-60 minutes after liquefaction[112]. Only after this natural process occurs can the sample be analysed, because the coagulum that forms right after ejaculation does not allow complete sperm motility and homogeneity in the sample, but after 60 minutes, sperm characteristics can start to wither[111–114,123,133].

It can take 15-60 minutes at room temperature for the sample to liquefy[113,123,125]. It is possible to place the container with the sample in a 37ºC incubator to expedite liquefaction, especially when it is not complete at 30 minutes after collection[112,114]. It is important to take note in cases where liquefaction is not complete after 60 minutes, as it may interfere with the semen analysis' results[112].

2.2.1.2 Volume

Besides providing information on the functionality of the sex accessory glands that produce the semen secretions, the measurement of ejaculate volume is important for the calculation of the total number of spermatozoa in the samples, as well of the number of non-sperm cells[112,123].

The recommended procedure for accurately measuring volume is sample weighting and assuming a semen density of 1 g/mL[112]. Although less precise, measurement with a graduated pipette can also be employed[112–114]. According to the latest WHO guidelines, a normal seminal volume is considered to be >1.4 mL[112].

2.2.1.3 Viscosity

The WHO recommended procedure for measuring semen viscosity is by aspirating the sample into a wide, disposable pipette, allowing it to drop by gravity and observing its behaviour[112,123]. If small drops are formed, viscosity is normal, but if a

thread with >2 cm forms, the sample is characterized by high viscosity[112]. It is important to ensure that the sample is liquified before this assessment, so that the absence of liquefaction does not interfere with viscosity evaluation[113].

High viscosity, similarly to non-liquefied ejaculate, impedes the homogenous distribution of sperm cells throughout the sample, and can impact sperm concentration and motility evaluation[112,113,123]. When that is the case, a narrow needle can be used to obtain representative aliquots for concentration assessment[113,133].

2.2.1.4 Appearance

Ejaculate appearance is a purely indicative parameter, that can be evaluated by inspecting the sample through the plastic container[114]. A normal sample should have a cream or greyopalescent appearance, that is usually opaque in cases of high sperm concentration, and clearer in cases of low sperm concentration[112]. Additionally, a red or brown tone may indicate the elevated presence of red blood cells[123].

The sample may contain gelatinous bodies, that besides interfering in the analysis, do not appear to have any clinical significance[112,114].

2.2.1.5 pH

It is important for the measurement of pH to be performed at 30 minutes after collection, but no longer than 1 hour, as the pH gradually increases in the sample due to CO_2 loss, which can result in a decline in semen quality[112,114,134]. The pH reflects the acidity/basicity characteristics of accessory glands secretions, and its reference value is 7.2[112,123].

The measurement should be performed with pH paper, on which a drop of the semen sample is placed, and the resulting colour is compared with the calibration strip[112,123].

2.2.2 Microscopic

While macroscopic evaluation mainly reflects the functionality and secretions of accessory glands, microscopic evaluation allows for a spermatogenesis-focused analysis[111]. To guarantee reliable and accurate results, it is essential to ensure that the aliquots are representative of the entire ejaculate, exhibiting the importance of giving enough time for liquefaction to occur, and of mixing the sample before taking aliquots[112].

2.2.2.1 Motility

Motility assessment relies on a fresh wet-mount slide preparation, which should be looked at under a phase-contrast light microscope immediately after liquefaction[112,113], because sperm motility is influenced by changes on pH or temperature, and by time to processing[85,123,134,135]. The recommended procedure is to place at least 10 µl of sample onto a microscope slide, preferably warmed to 37ºC, in which a coverslip is dropped into without forming air bubbles, followed by microscopic evaluation at 100x-200x magnification[112]. Subsequently, spermatozoa are counted with a multikey-counter, and the obtained values will be then expressed as percentages[114]. To increase the accuracy of the results, at least 200 sperm cells should be counted, scanning at least 5 different fields of the slide, preferably far from the edge of the coverslip[112–114].

According to the latest WHO guidelines, sperm motility can be categorized into 4 types (**Fig. 1**)[112,126]:

> A: fast progressively motile;
> B: slow progressively motile;
> C: non-progressively motile, in which there is tail movement, but no progression of the sperm cell;
> D: immotile, in which there is no tail movement.

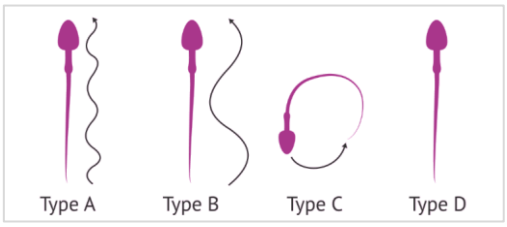


Figure 1: The four categories of sperm motility, defined in the sixth edition of the World Health Organisation laboratory manual[112]. Adapted from Álvarez et al., 2023[136].

The most recent reference values from WHO are at least 42% of total motility (fast progressively motile + slow progressively motile + non-progressively motile), at least 30% for progressive motility (fast progressively motile + slow progressively motile), up to 1% of non-progressive motility and up to 20% of immotile spermatozoa in a normal sample[112].

Motility, and more specifically, progressive motility, has been associated with successful IVF[76,137], enhancing its clinical significance and evaluation[114].

2.2.2.2 Concentration

There are a variety of options that allow sperm counting, but the Neubauer haemocytometer is the standard device[111,113,114]. This counting chamber requires that at least 50 µl of sample are diluted in a fixative solution to immobilize spermatozoa, making the counting process easier and more accurate[112,123]. According to WHO, the employed dilution for accurate assessment is dependent on the number of spermatozoa observed in a microscopic field, such as one where motility was evaluated[112]. Subsequently, 10 µl of solution should be loaded into the chamber, and enough time should be given to allow sedimentation of cells before counting[112].

The gridlines of a Neubauer haemocytometer are divided into nine main grids (**Fig. 2A**), in which the central grid is the one where sperm are counted to determine the concentration of the sample. This central grid is composed of 25 squares, each one subdivided into 16 smaller squares (**Fig. 2B)**, enabling the counting of sperm within their limits at 200-400x magnification[123]. The triple lines separating each of the 25 squares are important for boundary purposes, to guarantee that each spermatozoon is only counted once, to prevent overestimation[114]. Spermatozoa should only be included when both a head and a tail can be observed (**Fig. 3A**)[112].

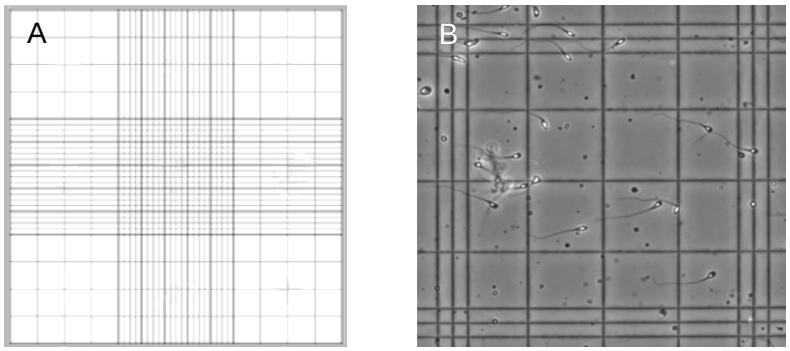


Figure 2: (A) Illustration of the grid lines on a Neubauer haemocytometer, conveying the central grid and the peripheral grids. **(B)** Example of sperm counting in one of the squares of the central grid. Adapted from World Health Organization, 2021[112].

It is important to count both the spermatozoa per mL (concentration) and the number of spermatozoa per ejaculate (total sperm count), obtained by multiplying concentration by ejaculate volume. The reference values for these parameters are 16 x 10^6/mL and 39 x 10^6, respectively[112].

It has been reported that the total sperm count is a more adequate way of portraying testicular sperm production[112,126]. However, an increase in both parameters

separately has been associated with a shorter time to pregnancy and a higher fertilisation rate[76,138,139].

2.2.2.3 Morphology

For this evaluation, a slide with a drop of liquefied sample should be smeared and stained, then observed at 1000x magnification with brightfield microscopy and oil immersion[112,113,123]. Using a multikey-counter, at least 200 sperm cells must be counted from several fields, to improve accuracy[112,113]. When distinguishing between morphologically normal and abnormal sperm cells, head, midpiece and tail features should be kept in consideration[112,125].

There are different morphology criteria that can be used for morphology assessment, but the latest WHO recommendation is to apply Kruger's strict criteria, allowing for a less subjective and more repeatable approach[112,140,141]. According to these criteria, all sperm cells that are not clearly morphologically normal (**Table 2**, **Fig. 3B**) or are borderline should be considered abnormal[112,141,142].

Table 2: Characteristics of a morphologically ideal sperm cell[112,141].

Head	Smooth, regular outline, oval shape; Defined acrosomal region, corresponding to 40-70% of the head; Absent of large vacuoles.
Neck and midpiece	Slim; Equal length compared with the head; Aligned with the major axis of the head.
Tail	Uniform diameter; Thinner than the midpiece; Length of about 10x the head.
Cytoplasmic residue	Less than ⅓ of head size.

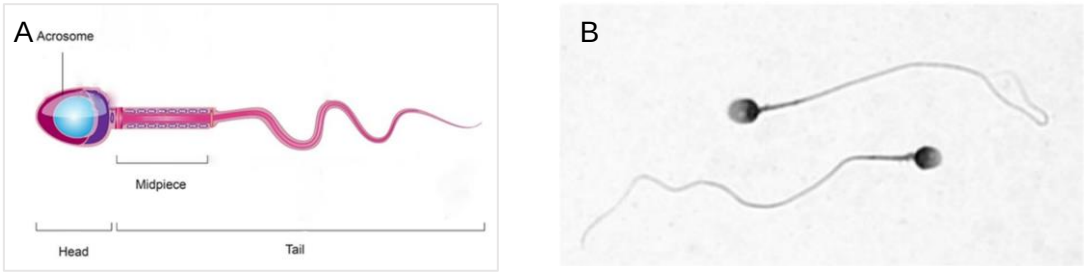


Figure 3: (A) Illustration of mature sperm structure. **(B)** Normal morphology spermatozoa. Adapted from Ahmadkhani et al., 2022 and Auger at al., 2016, respectively[143,144].

The use of strict criteria for defining normal and abnormal sperm morphology has been supported by the notion that it provides a prognostic tool for reproductive

ART outcomes[112,113,142]. Namely, it has been reported that the FR is significantly affected when the sperm morphology is below 4-5%[145–147]. Therefore, morphology is a sperm parameter that is highly related to sperm quality and fertilizing potential[112,123,140]. Additionally, it also encompasses diagnostic value, as sperm abnormalities are usually derived from defective spermatogenesis or spermiogenesis, or genetic abnormalities[112,123,148,149].

According to the latest WHO guidelines, sperm abnormalities can be divided into four categories: head (**Fig. 4A**), neck and midpiece (**Fig. 4B**), tail (**Fig. 4C**) or excess residual cytoplasm (**Fig**. **4D**) (**Table 3**)[112]. After counting, the percentage of normal morphology sperm in the sample can be calculated and its quality evaluated, given that the most recent reference value is >4%[112].

Table 3: Examples and impact of morphological abnormalities in spermatozoa on Assisted Reproductive Technology outcomes[112,123]. FR- fertilisation rate; ROS- reactive oxygen species; SDF- sperm DNA fragmentation; WHO- World Health Organization.

Head	Round, elongated, amorphous, pyriform or non-oval shape; High quantity of vacuoles; Double heads.	Irregular head shape has been associated with SDF[150–153], while a large head has been related to polyploidy and aneuploidy[154,155]. The presence of large vacuoles has also been associated with SDF, as well as poor embryo development[153,156]. One study also correlated head defects with poor embryo development[157], and another with decreased FR[150]. According to WHO, head defects are the ones that are more consistently associated with infertility[112].
Neck and midpiece	Thin or thick; Irregularly shaped; Bent; Not aligned with the major axis of the head.	A bent midpiece has been correlated with SDF[152]. Combined midpiece defects have also been associated with poor embryo development[157] and lower fertilizing potential[158].
Tail	Sharply bent; Coiled; Short; Irregular diameter; Multiple tails.	A coiled tail has been associated with poor embryo development and implantation[157].
Excess residual cytoplasm	More than ⅓ of head size.	It has been reported that sperm with excess residual cytoplasm are associated with higher DNA damage, due to elevated levels of ROS[149,159].

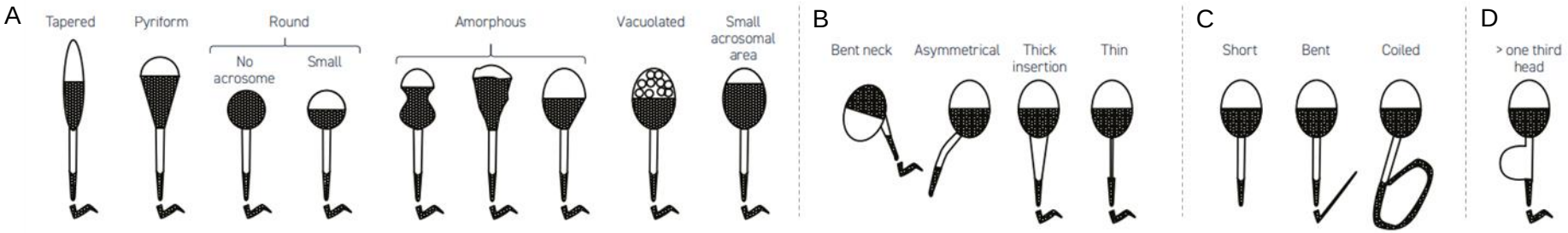


Figure 4: Illustrated examples of sperm cells' morphological abnormalities. **(A)** Head defects. **(B)** Neck and midpiece defects. **(C)** Tail defects. **(D)** Excess residual cytoplasm. Adapted from World Health Organization, 2021[112].

2.2.2.4 Agglutination

Agglutination is characterized by a clustering of motile sperm cells, while aggregation refers to a clustering of sperm cells with other non-sperm cells or between immotile sperm cells[112]. Their presence in the sample can be assessed in the same slide as motility, preferably at 100x magnification[112]. Agglutination is more relevant, as it may be related to immunological infertility, where there are anti-sperm antibodies present[111,112].

When agglutination is present, the sperm cells can be connected head to head, tail to tail, or in a mixed manner, with various degrees of severity (**Fig. 5**)[112,123]. Regarding the semen analysis, agglutination may hinder the accurate assessment of sperm concentration and motility[112,123]. *In vivo*, it affects the fertilisation potential by obstructing the interaction with the oocyte[34].

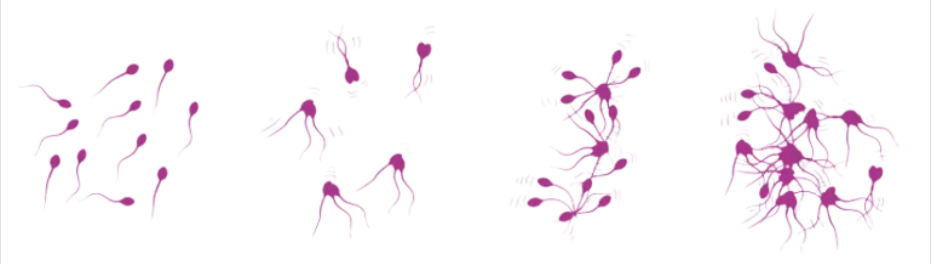


Figure 5: Illustration of different degrees and types of sperm agglutination. Adapted from Gilabert et al., 2021[160].

2.2.2.5 Vitality

In general, vitality evaluation is not routinely performed during a semen analysis, since its benefit is mainly seen for cases where there are ≥60% of immotile spermatozoa in the sample[112–114]. In such cases, it is important to evaluate the proportion of immotile dead *versus* immotile viable sperm cells, to get an idea of the availability of spermatozoa for ART, primarily ICSI[113].

The vitality assessment requires that a drop of liquefied sample is stained and smeared, and later observed at 1000x magnification with brightfield microscopy and oil immersion[112]. Similarly to the morphology evaluation, at least 200 sperm cells must be counted with a laboratory counter for optimal accuracy, and the results should be expressed as percentages[112]. In this case, the recommended method for staining is the eosin-nigrosin test, that discriminates between sperm cells according to their cell membrane integrity, through dye exclusion[112]. This means that only dead sperm cells are stained, showing red/pink heads, while live sperm cells are left unstained, with

white heads, which are easily distinguishable against the dark background, provided by the nigrosin in the solution (**Fig. 6**)[123,161]. The reference value for vitality is at least 54% of live sperm cells[112].

Figure 6: Example of eosin-nigrosin staining on a seminal smear, in which dead sperm cells have their heads stained red/pink, and live sperm cells are unstained. *Live sperm cells. Adapted from Agarwal et al., 2016[161].

2.2.2.6 Non-sperm cells

The presence of non-sperm cells in the sample, such as endothelial, epithelial or white blood cells can be observed in a counting chamber or on a slide[113]. A high number of epithelial cells is related to a substandard collection[114]. An elevated presence of “round cells”, encompassing white blood cells and immature sperm cells, is an indicator to proceed with calculation of the concentration of these cells in the ejaculate[112]. Specifically, an elevated presence of neutrophil leukocytes may be a marker for infection or inflammation within the male genital tract[48,112]. Furthermore, it may impact semen motility and DNA integrity by elevating ROS in the semen, with negative impact on its fertilizing potential[112,162,163].

The stained slide used for morphology can be used to count these cells, which are distinguishable by their nuclear morphology[112,113]. The threshold value for a normal presence of neutrophil cells in the sample is lower than 1.0×10^6/mL[112].

2.2.3 WHO Manual reference values

Since 1980, five revisions of the “Laboratory Manual for the Examination and Processing of Human Semen” have been published by WHO, with the most recent, the sixth edition, published in 2021[26,164]. The relevance of this manual is reflected in its effort to standardize the methods of semen sample analysis used in andrology laboratories[26,27,165]. In addition, they are also important in setting reference values (**Table 4**) of sperm parameters in fertile men, in order to assist the interpretation of

values obtained in semen analysis[113,165]. As a result, the manuals have supported the evaluation of male reproductive function, as well as the treatment choice in infertile couples[165].

Table 4: Lower 5th centile and its 95% confidence intervals of data for semen parameters in the reference fertile population, with time to pregnancy (TTP) by natural conception inferior to 12 months of regular, unprotected sexual intercourse[112,166].

	6th edition (2021)
Semen volume (mL)	1.4 (1.3-1.5)
Sperm concentration (x 10^6/mL)	16 (15-18)
Total sperm number (x 10^6)	39 (35-40)
Total motility (%)	42 (40-43)
Progressive motility (%)	30 (29-31)
Non-progressive motility (%)	1 (1-1)
Immotile spermatozoa (%)	20 (19-20)
Normal forms (%)	4 (3.9-4.0)
Vitality (%)	54 (50-56)

Although the existence of these values in the reference population is of undeniable significance and utility, it is important to note that the fact that the results of a semen analysis are above or below the reference values indicated by WHO does not necessarily mean that the patient in question is fertile or infertile, respectively[26,117,165], since these values do not represent a threshold between fertility and infertility, as brought to attention by WHO[112].

The general consensus seems to be that a single sperm parameter does not have diagnostic value and is insufficient in defining males' fertility potential or predict reproductive outcomes[44,117,123,126]. For that goal, sperm parameters must be interpreted collectively, while also considering each individual's clinical background[111,167].

2.2.3.1 Diagnosis

According to the obtained semen parameters and the WHO reference values, the sample can be classified into different categories (**Table 5**).

Table 5: Nomenclature of semen samples, according to the semen parameters[4,44,112]. WHO- World Health Organization.

Asthenozoospermia	% of progressively motile sperm below the lower reference limit defined by WHO.
Asthenoteratozoospermia	% of progressively motile and of normal morphology sperm below the lower reference limit defined by WHO.
Necrozoospermia	Absence of live spermatozoa in the sample.
Normozoospermia	Sperm concentration, % of progressively motile and morphologically normal sperm equal or above the correspondent lower reference limits defined by WHO.
Oligospermia/Hypospermia	Semen volume below the lower reference limit defined by WHO.
Oligozoospermia	Sperm concentration below the lower reference limit defined by WHO.
Oligoasthenozoospermia	Sperm concentration and % of progressively motile sperm below the lower reference limits defined by WHO.
Oligoasthenoteratozoospermia	Sperm concentration and % of morphologically normal sperm below the lower reference limits defined by WHO.
Teratozoospermia	% of morphologically normal sperm below the lower reference limits defined by WHO.

These categories aid the diagnosis of the couple's infertility, and may lead to investigation of the female factor, in cases of normozoospermia, or of further investigation of the underlying male factor in the other cases[123].

2.3 Semen processing

The success of ART treatments is highly dependable on sperm quality[143,168]. The sperm cohort in the ejaculate is marked by heterogeneity, so the selection of the most motile, mature and functionally normal sperm is essential for ART efficiency[7,168]. When the purpose is the processing for use in ART, the aim is to obtain the highest quantity of high-quality sperm from the raw semen, to increase the chances of successful outcome[112,113].

Therefore, the semen processing techniques should focus on concentrating motile spermatozoa while washing away the seminal plasma, debris, non-sperm cells and dead sperm cells[116,129,133]. An important note is that centrifugal force should be minimal, and when applied, should be as low as possible in order to produce a sperm pellet[113,114]. However, when sperm numbers are low (<10 x 10^6/mL), it is recommended to apply longer centrifugation time and/or higher centrifugal force, to maximize the recovery yield[112].

In the case of samples that are presumed to be azoospermic considering an initial wet-mount slide evaluation, the recommended course of action is to perform a centrifugation and subsequently examine the pellet. Only if no spermatozoa are found in the pellet resuspension can azoospermia be diagnosed[112,123].

The simplest step for semen processing consists of washing, that is based on centrifuging aliquot(s) of the sample twice[169]. Despite the negative consequences associated with centrifugation, namely related to ROS[114,170], it is a critical step to remove spermatozoa from the exposure to seminal plasma components that diminish the fertilisation potential[112,113,171].

2.3.1 Conventional techniques

The sperm isolation and selection methods that are most widely used are the swim up (SU) and the density gradient centrifugation (DGC) techniques[116,169]. These conventional methods are dependent on the inherent motility of the sperm cells[133].

Both methods provide a high yield of morphologically normal, progressively motile spermatozoa, with less DNA damage compared to the raw semen[172–174]. Nevertheless, there is no consensus regarding which of the two is better for processing normozoospermic samples when the recovery rates of viable spermatozoa and their parameters are compared, including DNA integrity[169,173–175], and even when the reproductive outcomes of ART cycles are compared[176,177]. However, when considering poor quality samples, it has been reported that DGC presents more advantages, indicating that the processing method should be chosen according to the samples' quality[169,177,178]. Additionally, it has been suggested that applying DGC and SU in the same sample might be the best strategy to obtain a higher recovery rate[169,179,180].

2.3.1.1 Swim Up

The SU procedure is the simplest and less costly of the sperm selection methods[115,133]. It consists of the migration of the progressively motile sperm cells out of the liquefied ejaculate (direct SU) or of the sperm pellet (washing + SU) and into the culture medium[113,116], leaving slow, immotile and dead sperm cells, as well as other cells behind[169].

For the migration, 0.5-1 mL of culture medium[116] is deposited on top of the sample aliquot/pellet, and the tubes are placed at 45º inclination, for 60 minutes on an incubator at 37ºC[112,133]. The inclination serves as a way to increase the surface area of the interface between the culture medium and the semen or pellet[114]. To increase recovery, it is possible to resort to multiple centrifuge tubes, thereby maximizing total interface area between the culture medium and the semen or pellet[113,114,133]. Afterwards, the upper portion of the media is collected, and concentration of the recovered spermatozoa is calculated[112,114,116].

2.3.1.2 Density gradient

DGC is based on the different densities of normal motile and abnormal sperm, as well as non-sperm cells[114,115]. This method can be employed with continuous or discontinuous layers[115,133]. The most common procedure is the discontinuous option, in which 0.5-1 mL of a 90% suspension of a colloidal silica is topped with 0.5-1 mL of a 40% suspension, on which the sperm sample is deposited[113,116]. To proceed to the separation of the cellular fractions, a centrifugation of 15-30 minutes is run, that being one advantage compared to the one-hour incubation period of SU[113,133]. Besides the difference in density, fast progressively motile spermatozoa actively swim through the gradient, leading to their deposition in the pellet first, while abnormal, immotile and debris form bands in the interphases along the gradient[113,115,133]. Therefore, after the centrifugation, only the pellet is collected, and resuspended for further use[114]. Additionally, a simple washing can be done, to guarantee that the spermatozoa resuspension is not contaminated with silica particles[115].

2.3.2 Advanced techniques

Sperm quality is a key factor for ART success[168] and this role has been progressively more emphasized[180], in view of the importance of genomic integrity of the sperm in embryo development and implantation[33,181]. The current sperm analysis performed in most andrology laboratories is a poor predictor of male fertility potential,

as often, men with normal semen parameters according to the WHO[112] can present low genomic quality[26,44,164,181]. Furthermore, a better evaluation of male fertility would be an asset in the decision of the most appropriate treatment in each case[182–184].

It is known that from the millions of sperm that are ejaculated, only a few hundreds arrive in the fallopian tube[185], which is indicative of the natural selection process that occurs in the female reproductive tract, that ensures that only the spermatozoa with fertilisation capability reach the oocyte[143,183]. Therefore, it is thought that it would be advantageous to mimic this strong selection as much as possible *in vitro*[143,169]. Theoretically, these techniques would enable the attainment of a pool of higher quality spermatozoa for treatments, that are more likely to give rise to positive outcomes[186], as it would decrease the risk of insemination (IUI and conventional IVF) or injection (ICSI) of oocytes with sperm of low genomic integrity[187].

It is highly relevant to look into alternatives for selection of functionally normal sperm to improve ART efficiency, especially in cases of male factor infertility, recurrent pregnancy loss or unexplained infertility[7,126,143,188]. Novel methods fall into different categories, such as based on hyaluronic acid binding, on magnetic cell sorting or on microfluidics[169]. Further research is needed for all the mentioned methods, as none of them are yet recommended for clinical routine application, for on-going debate regarding their efficiency on ART outcome[186] and so, they're not recommended to all patients despite the overall positive effect they have on enhancing sperm parameters[189–192]. Nonetheless, some authors recommend that the application of these techniques in specific cases may have a positive impact on reproductive outcomes[7,180].

2.3.2.1 Sperm DNA fragmentation

It has been reported that sperm DNA integrity plays a crucial role in reproductive success, both in natural and assisted reproduction[31,32,190]. The fact that infertile men have higher frequencies of DNA damage than fertile controls highlights the relationship between SDF and male infertility[32,181,193]. Furthermore, it has been shown that SDF is strongly correlated with suboptimal sperm count, motility and morphology[135,151,193].

It has been reported that SDF increases with paternal age, especially in men over 40 years old[72,73], and with paternal age on the rise[186], it has become a pressing concern. Additionally, a subtle male factor related to compromised sperm DNA quality with normal semen parameters may be associated with unexplained infertility[194].

Several tests with varying sensitivity and specificity can be used to determine the SDF in the sample[181], and a cutoff of 20-30% has been suggested for the minimal value at which chances of natural conception start to decrease when sperm chromatin structure assay (SCSA) is used[193,195]. However, the use of these tests is not yet recommended in routine clinical practice[112].

There have been contradictory reports on the effects of SDF on ART outcomes[181], but there have been reported correlations between the two. SDF has been associated with decreased PR, higher miscarriage rate, poor embryo development and quality, recurrent pregnancy loss and implantation or fertilisation failure, and it can be considered the most critical marker for sperm quality[32,112,181,188,190,196]. Furthermore, it may adversely impact offspring health[31].

It has been shown that the long incubation time and centrifuge steps included in conventional selection procedures may contribute to the increase of SDF in the sample due to the generation of ROS, affecting sperm viability[184,188].

2.3.2.2 Microfluidics

Microfluidics-based technologies for sperm preparation and selection have been a field of high interest[5,186]. Microfluidic sperm sorting (MSS) chips allow spermatozoa's selective migration through microchannels[32] at an optimized time-point[168], that isolates viable progressive spermatozoa from seminal plasma, nonmotile sperm, and other cellular debris[5,169], based on sperm motility[184]. MSS devices provide a more physiological strategy for sperm sorting[180], similar to the microchannels that form in the female reproductive tract through the cervical mucus, and guide sperm to the oocyte[133,183,197].

One major advantage is the lack of centrifugation steps, which results in minimal DNA damage in the selected sperm, when comparing to conventional techniques[169,180,182]. Additionally, the direct use of the sample means that it is a simple one-step procedure that is less time consuming[180], with high repeatability due to standardization, showing clear operational advantages[197,198].

MSS platforms can be employed resorting to a passive or active method[5], in which the latter utilizes external stimulators that can be based on rheotaxis (fluid flow variations), thermotaxis (thermical gradient) or chemotaxis (chemical gradient), mechanisms that occur *in vivo* sperm selection, within the oviduct[7]. Even though using MSS chips with active sperm sorting can be expensive, because of the need for highly

controlled microenvironments, this multi-feature strategy may increase selection yield[143]. However, passive methods are safer and less-invasive, and their driving force is sperm motility[5,143].

The main disadvantage of MSS lies on the fact that it still requires further studies, in order to validate their usage in clinical practise[180]. Other disadvantages are the need for natural motility in the sperm sample, and the lower volume and concentration of sorted sperm, due to the higher selectivity[143,182]. However, given that most male infertility cases rely on ICSI treatment, the reduced number of recovered spermatozoa will still be sufficient[182,194].

Previous studies have shown that MSS devices have a significant impact on several sperm parameters, such as morphology, progressive and non-progressive motility and SDF[168,182,184,188,194]. Therefore, the use of this device at least ensures that higher quality sperm is being used for treatments, regardless of a significant increase in reproductive outcomes. Despite the overall positive effect on enhancing sperm parameters[190], there are discrepant results regarding the ART outcomes of its use[188]. The disparity between reported outcomes has been justified as being at least in part due to the lack of standardisation in protocols (e.g. inclusion criteria), which interferes with the ease of comparison between the results[180], and the importance of a large sample size for studies has also been highlighted[188].

In couples with unexplained infertility where sperm sorting was performed through MSS or conventional methods, significant improvements in embryo quality (EQ) and fertilisation rate (FR) have been reported, especially in couples with previous unsuccessful ART attempts, in which there were poor fertilisation or implantation rates (IR), or poor EQ[184,187,188,198]. Regarding pregnancy rate (PR), the reported results have only shown a non-significant increase[184,198]. Consequently, it has been suggested that it is highly relevant to explore the specific cases in which MSS may be a valuable resource for improving reproductive outcomes[168].

3. Female gametes

3.1 Collection

Usually, the ovary releases only one oocyte during ovulation in each cycle, and although it is possible to perform ART treatments with a natural cycle, it is rarely done due to its low efficacy[55,116,125]. This is because there is interest in increasing oocyte yield, to balance out inefficiencies in the ART procedure, so that the likelihood for pregnancy and cumulative live birth rate (LBR) is increased[55,113,199], as it is more likely to obtain a good quality blastocyst when there is a higher initial number of oocytes[125,200].

To induce the growth of multiple ovarian follicles, women undergo controlled ovarian stimulation[4]. It consists of a pharmacological regime that stimulates more than one follicle to develop and mature in the same cycle[55]. The ovarian response exhibits considerable variability across individuals and cycles[201]. However, there are multiple options available in terms of both the chosen medication and correspondent protocol, that can be tailored to the patient's characteristics[81,113]. During the stimulation process, the follicles' growth is closely monitored[125]. Once at least three follicles reach the appropriate size of ≥ 17-18 mm in diameter, final oocyte maturation is induced through the administration of a trigger[55,202].

It is important to denote that ovarian stimulation does not only precede IVF or ICSI, that require oocyte collection, but also IUI, to increase the likelihood of *in vivo* fertilisation[4,55]. The main distinction is that in the case of IUI, the goal is not to produce a high number of developing follicles, but rather only one to two dominant follicles[203].

Ovum pick-up (OPU) from the ovaries is achieved by ovarian follicular aspiration, that can be performed 34 to 36 hours after the administration of the trigger[40,55], so that enough time is given to allow for final *in vivo* oocyte maturation, while ensuring that the retrieval occurs prior to spontaneous ovulation[125,129].

During the OPU, the follicular fluid is aspirated from the follicles into a collection tube, that is maintained at 37°C[40,204]. The fluid is then inspected by an embryologist using a stereomicroscope within a class II biological laminar flow hood with a heated surface at 37°C, in order to identify and isolate the cumulus-oocyte complexes (COCs)[40,115,204]. Once located, COCs are washed in buffered medium covered in liquid paraffin, and then kept in plates that contain pre-equilibrated IVF culture medium also

covered in liquid paraffin (**Fig. 7A**), and incubated at 37°C in 5% CO_2, 5% O_2, until the next step in the procedure, either insemination or denudation[40,116,205].

The number of retrieved oocytes has been reported as a predictor of cycle success[199], with optimal number as an intermediate range of 6 to 10-15, as this has been shown to provide the highest chance of successful ART[206,207]. Although the retrieval of >15 oocytes provides the opportunity to perform more frozen embryo transfers, a negative impact on maturation and FR without a significant increase on the cumulative pregnancy and LBR has been reported[206–209]. Conversely, when ≤5 oocytes are retrieved, PR is significantly lower[208,209].

COCs are constituted by an oocyte surrounded by the corona radiata and the cumulus oophorus, composed of corona and cumulus cells, respectively[40,116,127] (**Fig. 7B**). The morphological appearance of these cells can provide insight into the oocyte's maturity[113,204]. When mature, the corona cells are radiating from the oocyte, and the cumulus cells are expanded[40,125]. On the contrary, when immature, the cumulus cells are compacted around the oocyte[113,204]. Furthermore, the COC can present dark, irregular and dense cells, in which case, the COC is likely to be atretic and the correspondent oocyte is nonviable[129,205].

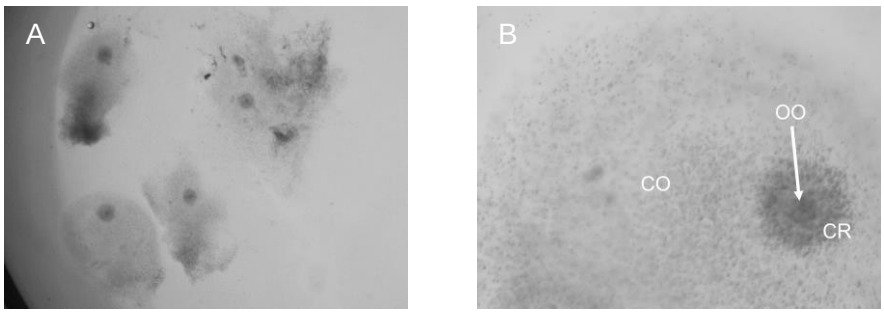


Figure 7: Cumulus-oocyte complexes in culture medium, after ovarian follicular aspiration. CO- Cumulus Oophorus; CR- Corona Radiata; OO- Oocyte. Images property of UMR (HSM).

However, the corona-cumulus expansion stage has been reported as a poor predictor of oocyte nuclear maturity and oocyte quality in stimulated cycles[205,210,211]. Thereby, this factor should be taken as a mere indicator of oocyte maturity[125].

3.2 Denudation

Denudation is the process through which the oocyte is stripped from its surrounding cumulus and corona cells, usually by a combination of chemical and mechanical procedures[113,115]. It is recommended to proceed to denudation 3-5 hours after OPU, to allow for cytoplasmatic maturity to be reached while the COCs are in

culture[125,212,213]. Due to their high sensibility to external factors, both COCs and stripped oocytes should be kept at pH 7.3 and 37ºC throughout the denudation process[113,115,204].

The chemical portion of the process is carried out in a medium with hyaluronidase enzyme, that acts on the hyaluronic acid present in the extracellular matrix of the cumulus oophorus[113,115,205], allowing for an initial dissociation of the cells[116]. Oocytes are exposed to repeated aspiration in this solution for only 30-40 seconds, to minimize the chance of damage[40,113,205]. Afterwards, the oocytes are carefully passed through pipettes of gradually decreasing diameter, to mechanically remove the remainder corona cells and complete denudation[116,204,205]. The pipetting is carried out in enzyme-free buffered medium, to guarantee that any trace of the enzyme is washed off[40,113,205].

Given the significance of oocyte maturity to ART success, only mature oocytes are selected for ICSI[116,214]. As such, the denudation procedure is essential to allow oocyte visualization and maturity status confirmation[113,127]. Additionally, it also guarantees that the surrounding cells do not interfere with the oocytes' microinjection[113,115]. Besides ICSI, denudation is also performed on oocytes prior to cryopreservation[113].

The oocytes are separated in the wells or microdroplets according to their maturity stage, and the culture dish with IVF culture media is incubated at 37°C in 5.0% CO_2, 5% O_2 until the next step in the procedure[40].

In the case of conventional IVF, denudation is performed 16-20h after insemination, to allow for confirmation of fertilisation[115,127,204]. However, given that sperm action has occurred, the cumulus cells are already looser, and only the mechanical denudation step is required[115,205,215].

3.2.1 Oocyte maturity

It is only after the completion of the denudation process that the oocyte becomes sufficiently visible to confirm its integrity and nuclear maturity stage, through morphological evaluation by light microscopy[40,113,205]. Therefore, it is only performed prior to ICSI or cryopreservation of the oocyte[216].

It is important to keep in mind that oocyte maturity is not only dictated by meiotic competence, but also by cytoplasmatic maturity[205,217]. However, in stimulated cycles, it is common for these two events to not be synchronized[125,218]. A mature cytoplasm is thought to be a crucial factor for correct embryo development[115,212]. Nonetheless, due

to the lack of clear morphological criteria for assessing cytoplasmic maturity, oocyte maturity is usually evaluated by its meiotic status[113,125]. At this stage, a mature oocyte is one that is ready for fertilisation, as oocytes only truly complete maturation when meiosis II is complete, with the extrusion of the second polar body, which only happens in consequence of oocyte activation, following fertilisation[40,113,205].

The presence of the first extruded polar body in the perivitelline space entails that the oocyte has finished meiosis I and is arrested in metaphase II (MII) (**Fig. 8A**), and thus, is mature[113,125,204]. From the collected oocytes, it is expected that around 80-85% will be MII mature oocytes[113]. Other scenarios that can be encountered are[113,204,205,219]:

> Immature oocyte in metaphase I (MI), shown by the absence of both the nucleus and the polar body in the perivitelline space (**Fig. 8B**);
> Germinal vesicle (GV) at prophase I, an immaturity state in which the nucleus is visible (**Fig. 8C**);
> Degenerated oocyte, in which the zona pellucida is empty or presents a fragmented oocyte.

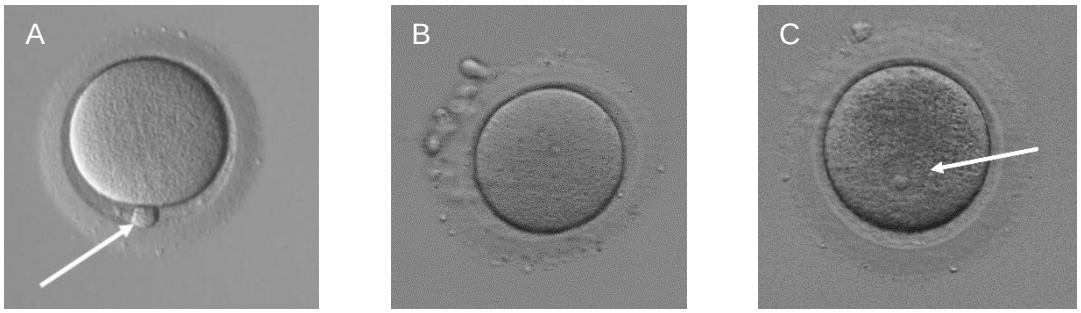


Figure 8: Oocytes in different maturation stages, after denudation. **(A)** Metaphase II oocyte, arrow showing the first polar body; **(B)** Metaphase I oocyte, with no visible nucleus or extruded polar body; **(C)** Germinal Vesicle, arrow showing the visible nucleus. Images property of UMR (HSM).

Usually, the GVs and degenerated oocytes will be discarded[113,205]. As for MI oocytes, there is the possibility that nuclear maturity will spontaneously be reached *in vitro* after denudation[113]. However, when such oocytes are injected, the resulting embryos may exhibit chromosomal abnormalities and decreased developmental competence, probably due to the absence of cytoplasmatic maturation[214,220].

3.2.2 Oocyte morphology

Similarly to maturity evaluation, oocyte morphology evaluation is only possible after denudation[115,205]. Since oocytes play a big role in fertilisation and embryo development[221–223], their morphology should be carefully considered[202,216].

After ovarian stimulation and follicle aspiration, a diverse pool of oocytes is obtained, with varying potential to develop into a good quality embryo and lead to a healthy pregnancy[205,217], especially because the stimulation process overrides the natural elimination of oocytes that would otherwise not develop[224]. It is crucial for the optimization of ART success to choose the oocytes with most developmental potential, to increase LBR and decrease miscarriage rates[217,221,225,226].

Although molecular criteria for this evaluation and selection are also studied, a simple non-invasive morphological evaluation using light microscopy remains the most popular method, despite its qualitative and subjective bases[202,216,217,226]. Following morphological criteria, the characteristics of an ideal MII oocyte (**Fig. 9**) are described in **Table 6**.

Table 6: Characteristics of a morphologically ideal oocyte[113,202,205,216,218,226].

Zona Pellucida	Spherical, clear, uniform
Perivitelline Space	Narrow, absent of granules or inclusions
First Polar Body	Single, intact, round or ovoid, in the PVS, size-appropriate
Cytoplasm	Spherical, translucent, clear or moderately granular, with no inclusions or vacuoles

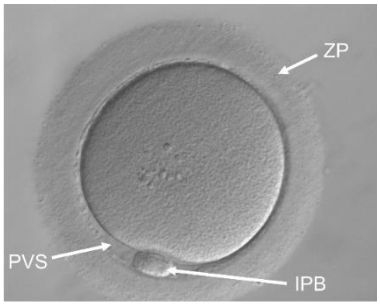


Figure 9: A morphologically ideal and mature (metaphase II) oocyte. IPB- First Polar Body; PVS- Perivitelline Space; ZP- Zona Pellucida. Adapted from the Atlas of Human Embryology- Chapter 1: The Oocyte[227].

It is thought that oocyte morphology is impacted in a multifactorial manner[217], by ovarian stimulation and response, follicular microenvironment, and intrinsic factors such as age or genetic background[113,221,224,228].

The morphological criteria can be divided into extracytoplasmatic and intracytoplasmatic abnormalities[205]. Extracytoplasmatic features include anomalies related to the zona pellucida, perivitelline space and first polar body, while

intracytoplasmatic features are the ones regarding possible abnormalities in the cytoplasm[202,205,216,224]. In **Table 7**, there is a summary of the impact of these parameters on ART outcomes.

Special attention must be given to two abnormalities that can be related to potential developmental concerns: giant oocytes and oocytes with clusters of smooth endoplasmic reticulum (sER). It is recommended for these oocytes to be closely monitored, and when possible, embryos deriving from sibling oocytes without these defects should be preferred for transfer[218,229].

Table 7: Impact of oocyte morphological abnormalities on ART outcomes. Underlined dysmorphisms are considered to have a more severe impact on oocyte quality, according to the Istanbul consensus workshop on embryo assessment[218]. ART- assisted reproductive technology; EQ- embryo quality; FR- fertilisation rate; IPB- first polar body; IR- implantation rate; OPU- ovum pick-up; PR- pregnancy rate; PVS- perivitelline space; sER- smooth endoplasmic reticulum; ZP- zona pellucida.

Extracytoplasmatic	**Zona Pellucida**	Darkness (**Fig. 10A**)	Contradictory results have been reported on whether a dark ZP has a significant impact on FR, IR, clinical PR, and EQ[216,230,231]. ZP abnormalities may arise from defects in folliculogenesis[216,225].
		Thickness (**Fig. 10B**)	A thinner ZP has been associated with a higher FR, probably due to easier penetration by the spermatozoa[202,232]. However, a thicker ZP has been associated with increased PR[233].
		Fractured	A fractured ZP may arise due to over-maturation of the oocyte, or in consequence to the aspiration technique during OPU, and although its occurrence has been associated with a decreased FR, it does not appear to have a significant impact on ART outcome[115,234,235].
	Perivitelline space	Size (**Fig. 10C**)	A large PVS is reported to have a negative impact on FR[228,236]. However, most studies report no impact on embryo development[202]. It is suggested that varying results may arise depending on the degree to which the size increases[113].
		Granularity (**Fig. 10D**)	It has been suggested that PVS granularity is a consequence of the stimulation with gonadotropins[228,237], though it is also hypothesised that the presence of cellular debris in the PVS may arise from folliculogenesis processes related to maturation[216,224]. There are contradictory reports on whether it negatively impacts EQ[228,238], but it has been shown that FR is not significantly impacted[228,237,239].
	First Polar Body	Size (**Fig. 10E**)	Large IPB has been associated with postovulatory oocyte aging and genetic mutations, with a negative impact on embryonic development[240] but not on FR[241].
		Integrity (**Fig. 10F**)	Contradictory results on a wide array of ART outcomes, such as FR, IR, EQ and PR[216,226,242–244]. Fragmentation may be caused by *in vitro* oocyte aging, and thus, it may not be considered a quality marker, or even an abnormality[224,242,243]. A degenerated PB may be related to oocyte maturity defects[245].

	Shape and size	Irregular shape (**Fig. 10G**)	It has been consistently reported that apart from ovoid shape, other shape irregularities do not seem to impact FR and EQ[225,236,238,239,246]. Although a defective cleavage pattern has been detected on ovoid shaped oocytes, it is still controversial whether it has an impact on cycle parameters and outcomes[247,248].
		Giant oocytes (**Fig. 10H**)	Considering that normal oocytes have generally 120 µm in diameter, oocytes with 200 µm or more in diameter are considered giant[115]. This abnormality arises rarely, and is associated with defects during oogenesis[249,250]. It has been reported that all giant oocytes exhibit chromosomal abnormalities and are usually diploid, resulting in polyploid embryos[205,249–251]. They pose as dangerous to ART treatments because despite the possible aneuploidy, they are capable of following normal embryonic development after normal or abnormal fertilisation[113,205,249]. Therefore, these giant oocytes should be discarded from treatments, as their use increases the probability for spontaneous miscarriages[225,249,250]. However, recovered sibling oocytes are shown to not have diminished quality, and can be used without negative implications in cycle success[252,253].
Intracytoplasmatic	**Colour**		There are contradictory results regarding dark oocytes' impact on FR and EQ[217,239,246]. Few studies focus on the study of this characteristic individually, as it is typically observed in conjunction with other abnormalities[225].
	Granularity		Granularity is a commonly observed cytoplasmic characteristic[115,216]. Generally, granularity may arise from an accumulation of cellular organoids[216], or be a signal of cytoplasmic immaturity[115,254]. It is important to distinguish between generalized (**Fig. 10I**) and centrally located granularity[115,216] (**Fig. 10J**), as different impacts are reported for these two cases. Homogeneous light or moderate granularity is usually not considered a dysmorphism, but a normal oocyte variant, as no impact on FR and EQ is consistently reported[205,216,236,238,239]. There are contradictory reports regarding dense granularity in the centre of the cytoplasm's impact on EQ[236,254], but consistent reports on its negative impact on pregnancy,

		miscarriage and live birth rates[254,255]. Consequently, some authors defend that this dysmorphism is indicative of poor oocyte quality[216].
	Vacuoles (**Fig. 10K**)	The presence of vacuoles in the ooplasm impacts cycle outcomes depending on their size and quantity[225]. Few or small vacuoles, such as vacuoles with 5-10 µm in diameter, have less impact on the cytoskeletal structure of the oocyte[218,256]. On the contrary, it has been reported that the presence of vacuoles with more than 14 µm of diameter is associated with fertilisation failure[256]. The absence of a significant impact on EQ has been consistently reported[228,236,238,239].
	sER (**Fig. 10L**)	Aggregation of sER within the cytoplasm has been associated with a negative impact on embryo development, EQ, IR and PR[238,257,258]. Additionally, an increased chance of miscarriage, foetal anomalies, obstetric complications and development of Beckwith-Wiedemann syndrome has been reported[238,257,259], although healthy live births have also been reported[260,261]. However, no significant impact on FR and EQ has been reported[228,236,260]. It is not established whether sER-negative oocytes from a cohort with sER-positive oocytes have a negative impact on outcomes as well[257,261]. It is recommended to prioritize the transfer of embryos derived from sER-negative oocytes[229].

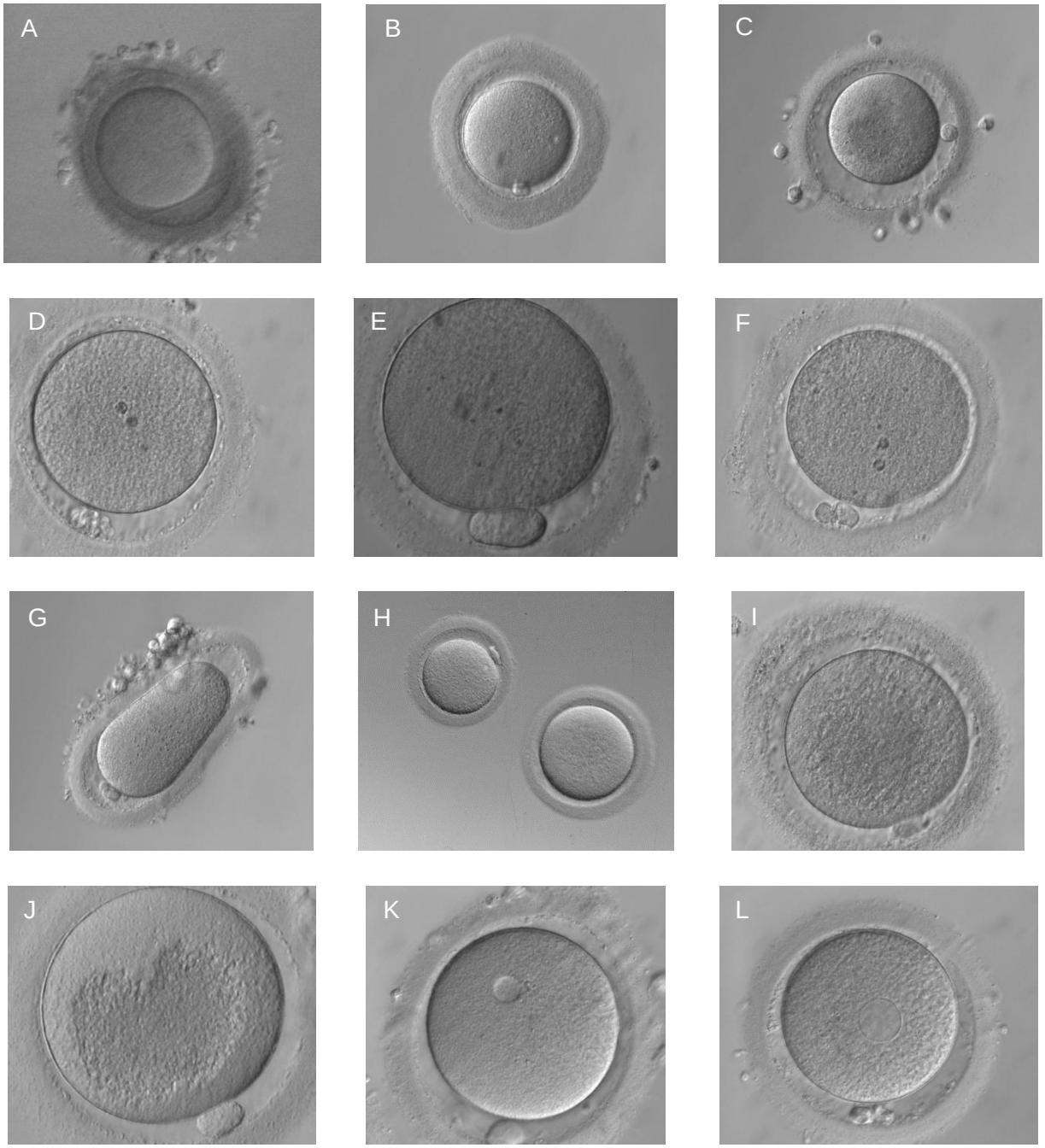


Figure 10: Examples of oocyte morphological abnormalities. **(A)** Dark zona pellucida. **(B)** Thick zona pellucida. **(C)** Big perivitelline space. **(D)** Granular perivitelline space. **(E)** Big first polar body. **(F)** Fragmented first polar body. **(G)** Ovoid mature oocyte. **(H)** Normal (left) and giant (right) oocytes. **(I)** Homogenous cytoplasmic granularity. **(J)** Central cytoplasmic granularity. **(K)** Small vacuole on cytoplasm. **(L)** Smooth endoplasmic reticulum cluster on cytoplasm. Adapted from the Atlas of Human Embryology- Chapter 1: The Oocyte[227].

There is still no consensus on which parameter is the best for oocyte selection[202], as the subjective and variable nature of the evaluation remains an obstacle to the establishment of specific criteria[205,216,217].

In stimulated cycles it is common for the majority of oocytes to present morphological abnormalities, which makes it difficult to study the impact of each

morphological deviation individually[216,228,236]. Furthermore, some deviations from the ideal oocyte can be attributed to phenotypic variability, having no impact on oocyte competence, while some morphologically normal oocytes may have decreased competence[113,202,216]. Therefore, oocyte morphology is not considered a predictive factor[216].

4. Assisted Reproductive Technology

4.1 Definition

Medically assisted reproduction (MAR) is a field of medicine that aims to treat infertility, with the ultimate purpose of achieving pregnancy in couples diagnosed with the disease. It includes all the necessary steps for the referred treatment, that range from ovarian stimulation, ovulation triggering, ART and necessary surgeries[4].

ART procedures are the methods used in MAR that require *in vitro* handling of human oocytes, sperm, or embryos, in the context of achieving conception. Examples of the included techniques are *in vitro* fertilisation, intracytoplasmic sperm injection, embryo transfer (ET) and sperm, oocyte and embryo cryopreservation, among others[4].

Since 1978, the year of birth of the first IVF baby[116], it is estimated that more than 12 million babies have been born as a result of ART treatments up until 2022[262].

4.2 Intrauterine insemination

IUI is considered a first-line treatment for infertility[116,129,263], that consists of the placement of a processed semen sample in the uterine cavity, timed to coincide with ovulation[126,264,265]. This procedure overrides some of the sperm selection steps within the female reproductive tract by bypassing the cervix, resulting in a higher motile spermatozoa number near the oocyte(s), therefore facilitating *in vivo* fertilisation[129,265,266].

IUI is advised in cases of ovulatory dysfunction, unexplained infertility, mild male factor, cervical factor, mild endometriosis, partial tubal factor, or ejaculatory/coital factors, but contraindicated in cases of severe endometriosis, bilateral tubal factor or severe male factor[129,264,266,267].

It is possible to perform IUI with a natural cycle, or by relying on ovarian stimulation[126] to induce the development of up to two follicles[40,129,268] and increase the chances of fertilisation, while also guaranteeing insemination timed with ovulation[265]. Natural cycles are more suitable for couples with ejaculatory/coital or cervical factors[116]. When more than two mature follicles are observed, the cycle should be cancelled, due to the risk of a multiple pregnancy, that should be avoided in ART treatments[264,265].

The LBR is significantly higher in stimulated rather than natural cycles[269,270]. When considering IUI cycles with autologous sperm, the overall PR and LBR are around 12% and 8%, respectively[271,272], although these values vary depending on the infertility aetiology[240].

Regarding the semen sample, it should be collected on the day of the IUI, and processed as described in section 2.3. The sample is kept at 37ºC in between processing and IUI[129]. At the time of IUI, usually 32-36h after the trigger, 0.2-0.5 mL of the processed sample are loaded into a catheter, that will be used by the gynaecologist to proceed with uterine insemination[129,266].

IUI is simpler, cheaper and less invasive than IVF[40,263], however, it presents lower success rates and an association with multiple pregnancies[265,266]. It has been reported that female age and total motile sperm count are the most important parameters for the success of IUI, along with the clinical monitoring of the patient[40,129]. Considering reported results, it seems that IUI does not present as a valuable treatment for women over 40 years old, compared to IVF[60,265,273].

4.3 *In vitro* fertilisation

IVF is carried out by co-incubation of COCs with spermatozoa, enabling a natural fertilisation process to occur *in vitro*[126,127,205].

The main aetiology for which IVF is indicated is tubal factor infertility, but mild male factor, endometriosis, unexplained infertility, diminished ovarian reserve or previous unsuccessful IUI cycles are also indications[125,129]. On the contrary, severe male factor or the use of cryopreserved oocytes are contraindications[129,215].

The oocytes are obtained as described in section 3.1, and the sperm sample is processed as described in section 2.3. The ideal timing for insemination to take place is around 5 hours after OPU, which should correspond to 40-41 hours after trigger administration[205]. This period between OPU and IVF is essential for the oocyte's maturation process[205].

The fundamental principle of IVF is to increase the probability of sperm-oocyte interaction, in a dish with pre-equilibrated fertilisation medium covered with paraffin oil[40,115,129,205]. For that purpose, 50 000-100 000/mL of motile and morphologically normal spermatozoa are placed in the dish with up to five COCs[40,116,125]. Afterwards, the dish is incubated at 37ºC and 5% CO_2, 5% O_2, usually for around 17 hours[40,115,129].

After that period of time, denudation can take place, followed by a fertilisation check and transfer to fresh culture medium[40,205,215].

It has been reported that this prolonged co-incubation period of 16-18 hours may have negative implications on the oocyte, due to the extended exposure to metabolic waste products, particularly ROS derived from the sperm cells, especially in male factor patients[215,274,275]. Consequently, a significant improvement on IR, PR, EQ and embryo development has been reported when a 1-2-hour incubation period is employed, compared to prolonged incubation[274,275]. However, contradictory results have also been published[276,277]. As such, there is no consensus on whether the co-incubation period should be shortened[129].

The number of inseminated spermatozoa may vary between cycles and patients. Increasing this number may be employed as a strategy in cases where previous IVF cycles have failed or when sperm morphology falls below the reference value, and reducing this number can be applied in cases where previous IVF cycles presented an elevated rate of polyploid fertilized oocytes[113,125,129,204,205,278].

The IVF procedure is less invasive and simpler than ICSI, and still encompasses natural sperm selection and fertilisation[205]. However, it relies on inherent sperm motility, and is therefore unsuitable for use in cases of severe male factor infertility[129,205].

The overall PR and LBR for IVF cycles are reported to be around 25% and 20%, respectively[272]. Several factors influence these success rates, namely the female age, the response to stimulation and the infertility diagnosis and duration[125,129,279]. Compared to IUI, IVF has higher success rates in women over 40 years old[60]. Furthermore, it has been reported that for women over 40 years old with non-male factor infertility, IVF is preferred over ICSI, as an increase in outcomes is not noted when the latter is employed[280].

4.4 Intracytoplasmic sperm injection

ICSI describes the procedure in which a single spermatozoon is selected and microinjected directly into a single mature oocyte's cytoplasm[40,116]. This ART procedure bypasses natural sperm selection[40,129].

The main indications for ICSI are a low FR (<30%) or total fertilisation failure (TFF) following IVF, or severe male factor- oligozoospermia, asthenozoospermia, teratozoospermia and other possible combinations[114,125,127]. Additional factors that

require the use of ICSI are the resort to cryopreserved oocytes, as well as testicular or epididymal sperm cells in azoospermia cases[113,125,127,129,215]. Although ICSI may be employed in cases with other aetiologies, its use in non-male factor cases has been shown to not confer advantages over IVF regarding live birth outcomes[281–283]. Therefore, ICSI for non-male factor infertility is advised against[281–283].

Prior to the ICSI procedure, the oocytes are retrieved and denuded, as described in sections 3.1 and 3.2, respectively. Once again, similarly to IVF, the semen sample should be processed according to section 2.3. The time interval between denudation and ICSI has been shown to impact FR and PR[40,284]. It is recommended that ICSI is performed within 4 hours after denudation[284], not surpassing 40h post-trigger administration, to avoid postovulatory oocyte aging[114,205].

ICSI can be divided into four main steps, the first one being the denudation of the COCs. The following three steps are: selection and immobilization of a viable spermatozoon; correct positioning of the oocyte; and rupture of the oolemma followed by release of the sperm cell into the oocyte's cytoplasm[115,127].

The selection of a single viable spermatozoon within the pool of processed sperm cells by the embryologist is dependent on a morphology and motility visual evaluation, with the goal of selecting a functional sperm cell, capable of achieving fertilisation[40,115,129]. However, the use of these indirect sperm function markers may result in the injection of poor-quality spermatozoa[129].

The evaluation is facilitated by the use of polyvinylpyrrolidone (PVP) microdroplets covered with oil in the injection dish, on which an aliquot of the processed sperm solution is placed on[113,114,125]. The viscosity of this solution deaccelerates spermatozoa and prevents sticking to the interior of the pipette, allowing for an easier selection, immobilization, and overall handling[40,113,127].

Although a normal morphology does not always correspond to the absence of DNA damage[285,286], better ICSI outcomes have been reported when morphologically normal spermatozoa are used, compared to the injection of abnormal sperm cells[287,288]. As described in **Table 3** (section 2.2.2.3), sperm morphology abnormalities have been linked to poor sperm function and ART outcomes[129]. Regarding motility, a motile sperm cell is preferentially chosen for injection over immotile spermatozoa[129]. However, there may be cases, more commonly in surgically retrieved sperm samples, in which there are few or no motile or "twitching" sperm cells to choose from[129]. In those cases, an immotile sperm cell can be injected, although its use has been related to worse ICSI

outcomes[289,290]. Alternatively, in cases of poor sperm quality, it is also possible to place elongated drops of culture media in the injection dish, on which an aliquot of the processed sperm solution is placed on, followed by a small period of incubation[114]. This allows for motile sperm to swim out of from the cellular debris and nonmotile sperm, facilitating the search[113,114].

Immobilization of the chosen spermatozoon is essential for correct oocyte activation, and consequently, fertilisation[115,291]. This immobilization is usually achieved through a mechanical technique with the pipette, by applying pressure to the tail, below the midpiece, against the bottom of the dish with a swift motion (**Fig. 11A**)[113–115]. The resultant rupture of the tail's plasma membrane enables the release of sperm cytosolic factors, that take part in the oocyte activation process[115,291,292].

The ICSI apparatus is made up of an adapted inverted microscope, that includes a heated surface at 37ºC and two micromanipulators, one on each side of the microscope[113,114,127]. These micromanipulators allow for fine and coarse three dimensional control of movement of the holding and injection pipettes, on the left and right sides, respectively[114,115,127]. The injection pipette is used for aspirating, immobilizing and injecting the sperm cell, while the holding pipette's role is to hold the oocyte during the procedure[40,114,127]. Proper preparation of the equipment prior to injection is crucial, as well as the preparation of the injection dish with equilibrated medium microdroplets covered in paraffin oil[114,127].

Each oocyte is placed on an individual medium microdroplet on the injection dish[127]. For the injection, the oocyte must be correctly placed on the holding pipette, where it is held in place via gentle suction[113–115]. In order to minimize meiotic spindle disruption during the procedure, the oocyte should be positioned so that the polar body sits at the 6 or 12 o'clock position (**Fig. 11B**), and the injection pipette should enter the oolemma in the 3 o'clock position[40,127,293] (**Fig. 11C**).

Before the injection itself, the chosen and immobilized spermatozoon should be aspirated into the injection pipette tail first, while minimizing the aspirated medium[40,114,127]. After aligning this pipette in the same focal plane as the oolemma, the sperm cell can be brought closer to the tip. Subsequently, the injection pipette should be carefully advanced into the zona pellucida, and gentle suction can be applied to allow the rupture of the oolemma (**Fig. 11C**). Afterwards, the sperm cell, along with the ooplasm backflow, can be ejected into the ooplasm and the pipette carefully withdrawn from the oocyte[113,114,127] (**Fig. 11D**). The injected oocytes are then cultured at 37° C and 5% O_2, 5% CO_2[127].

It has been reported that the type of oolemma rupture is associated with ICSI outcomes, with a flexible oolemma in which minimal suction is used being the ideal scenario in terms of oocyte survival and FR[294,295].

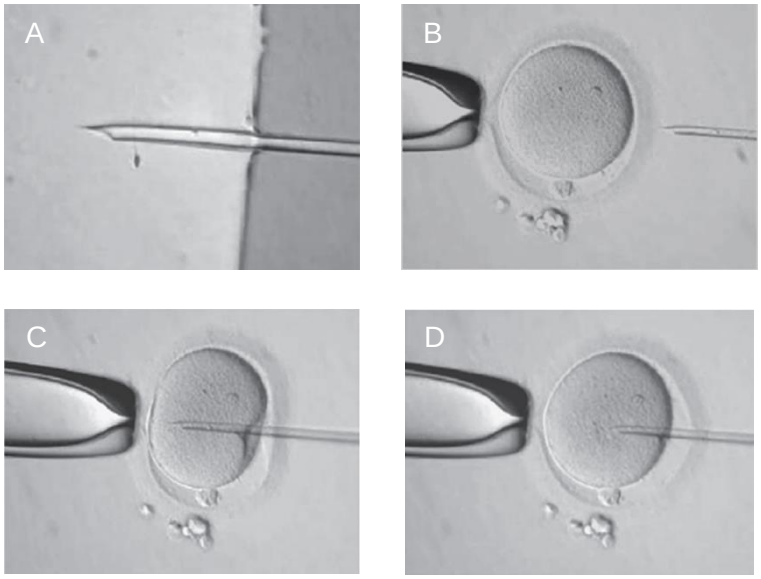


Figure 11: Intracytoplasmic sperm injection steps. **(A)** Immobilization of the selected spermatozoon by applying pressure to the tail against the bottom of the injection dish, with the injection pipette. **(B)** Correct positioning of the mature oocyte on the holding pipette, with the polar body at the 6 o'clock position. **(C)** Injection pipette entering the oolemma in the 3 o'clock position, with rupture of the oolemma aided by slight suction. **(D)** Withdrawal of the injection pipette, after ejection of the sperm cell into the oocyte. Adapted from Bormann, 2014[127].

Although ICSI enables severe male factor patients to become biological parents, it is important to consider the potential negative implications associated with bypassing numerous biological steps and the high level of *in vitro* manipulation of both oocytes and sperm cells involved[129,205]. While there is no widely recognized consensus, the majority of studies have found no significant difference between children conceived through ICSI and those conceived naturally with respect to growth, chronic illnesses, neurodevelopment and overall psychological well-being[129,296–299]. However, there is a potential increased likelihood that ICSI-conceived offspring may inherit underlying genetic abnormalities causative of severe male factor[300,301], evidenced by some reports on poorer semen quality on male ICSI children[302,303].

Furthermore, ICSI is more costly, invasive, labour-intensive and time-consuming compared to IVF, requiring highly skilled operators[205,281,282].

The overall PR and LBR for ICSI cycles are reported to be around 23% and 17%, respectively[272]. Important improvements of ICSI in comparison to IVF are shown in the increase of FR and decrease in the incidence of unexplained TFF cycles[304,305].

4.4.1 Artificial oocyte activation

Although ICSI is utilized as a solution for low FR and TFF patients, there remains a low incidence of 1-5% of ICSI cycles in which no oocytes are fertilized[113,127,306]. It has been reported that the majority of these cases are caused by failure of oocyte activation[307,308]. This may occur due to inherent oocyte defects, or to sperm function failure[129,292,306].

Artificial oocyte activation (AOA) can be employed in subsequent cycles of ICSI with TFF or poor FR (<30%)[115,129,309]. The most common method through which this technique is employed consists of exposing the oocytes to a calcium ionophore solution after injection, followed by washing and placing into culture media[115,306]. The purpose of this method is to artificially induce an influx of Ca^{2+} into the ooplasm, and consequently stimulate oocyte activation[113].

The reported ICSI outcomes when this treatment is applied are highly variable, but it has been reported that the outcomes appear to differ depending on the underlying cause of previous TFF or poor FR[113,292,309]. Nevertheless, it is somewhat consistent that AOA yields significantly improved outcomes in cases of previous TFF or low FR cycles, in terms of FR, PR and LBR[309–311].

Preliminary reports vouch for the safety of this procedure, regarding birth defects and development[312,313], but concerns are still standing, as this is not yet an established and standardized procedure[115,313].

4.5 Fertilisation

The oocyte fertilisation after IVF or ICSI is confirmed through evaluation of the presence and number of pronuclei and polar bodies[115,215]. As mentioned in section 3.2.1, the meiosis II process of the oocyte is only complete after sperm penetration and fertilisation, resulting in the extrusion of the second polar body[113,125]. The two pronuclei (PN) correspond to the male and female haploid genomes, which will fuse to form a single diploid nucleus containing genetic material from both parents[115,116,125,205]. Therefore, the presence of two individual or fragmented polar bodies in the perivitelline space, and of two juxtaposed and similar-sized pronuclei (2PN) with visible nucleoli in the centre of the ooplasm are morphologic markers of normal fertilisation by a single sperm[125,205,215,218] (**Fig. 12B**).

In the case of conventional IVF, it is necessary to perform denudation of the cumulus cells prior this assessment (section 3.2), to allow for ooplasm microscopic

observation at high magnification[115,125,204]. Consequently, it is only at this step that oocyte nuclear maturity can be confirmed[125,215].

For both IVF and ICSI oocytes, the cells are transferred to fresh cleavage medium, and usually grouped according to their stage: degenerated, immature, unfertilized, 2PN fertilized or fertilized with less (**Fig. 12A**) or more (**Fig. 12C**, **12D**) than 2PN, and culture continues until the next step in the procedure[125,129,205,215].

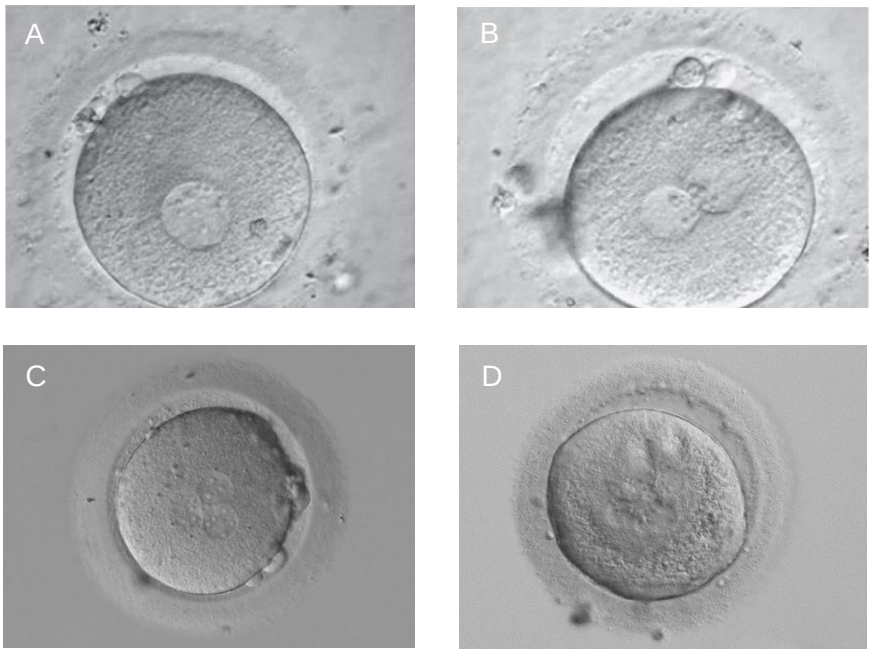


Figure 12: Possibilities of PN numbers at fertilisation assessment on day 1 after oocyte insemination or injection. **(A)** Unipronuclear zygote. **(B)** Normal scenario with two PN. **(C)** Tripronuclear zygote. **(D)** Multi-pronuclear zygote. PN-pronuclei. A and B adapted from Bormann, 2014[127]; C and D property of UMR (HSM).

The optimal timing for conducting the fertilisation check is 16 to 18 hours post-insemination or injection (hpi)[125,129]. It is imperative to ensure oocyte observation within the aforementioned time frame, to guarantee assessment before the two PN go through syngamy (merging of the PN) and mitotic divisions initiate[116,204]. Beyond this point, pronuclear observation is no longer possible[215,314]. Thus, fertilisation evaluation before 16 hours or after 18 hours of culture may lead to false conclusions, because incorrectly fertilized embryos may not be distinguishable from correctly fertilized ones[204,315,316]. The possible scenarios regarding number of PN and their meaning are described in **Table 8**.

Table 8: Possibilities of PN numbers at fertilisation assessment on day 1 after oocyte insemination or injection, and justifications for deviations from the normal scenario of 2 PN. ICSI- Intracytoplasmic sperm injection; IVF- *In vitro* fertilisation; PN- pronuclei; PVS- Perivitelline space.

0 PN	The absence of any PN is indicative of an unfertilized oocyte[307], or a fertilized zygote in which syngamy has already occurred (evidenced by the presence of two polar bodies)[218,317].	
1 PN	The presence of a singular pronucleus may be indicative of a haploid zygote[318]. This abnormal pronuclear number may be caused by parthenogenetic activation of the oocyte, an asynchronous formation of PN or abnormal syngamy[318–320].	
2 PN	The presence of two PN is indicative of a diploid zygote[115]. However, situations may arise where the zygote shows 2PN, but is triploid: when a diploid oocyte is normally fertilized, or a haploid oocyte is fertilized by a diploid sperm cell[321,322].	
3 PN	The presence of three or more PN is indicative of a polyploid zygote[115].	The presence of three PN is the most common multipronuclearity state[115]. When considering IVF oocytes, multiploidy may be caused by di- or polyspermic fertilisation[115,320,321], that can be correlated with high sperm concentration or oocyte aging[323]. In IVF or ICSI cycles, triploidy may also arise due to oocyte meiotic defects, leading to the retention of the second polar body, in which case, a single polar body is observed in the PVS[307,320,321]. A combination of these events may lead to tetraploidy[324].
>3 PN		

In the cases where an abnormal PN number is observed, the oocytes should be discarded[125,127]. The reasoning behind this is that there are associations with karyotypic irregularities, that may lead to spontaneous miscarriages and congenital malformations[325–327]. It has been reported that non-viable zygotes with abnormal PN numbers will most likely suffer developmental arrest in culture, while viable zygotes can go through regular development and lead to viable pregnancies[115,328].

To calculate FR, the number of correctly fertilised oocytes are divided by the total number of inseminated COCs (for IVF) or of injected MII oocytes (for ICSI)[127,215]. Usually, the FR for IVF and ICSI is of around 55-65% and 70-80%, respectively[327,329].

4.6 Embryo culture

One of the purposes of ART is to produce high-quality viable embryos, that can be chosen for ET, thereby maximizing the chances of achieving pregnancy and live

birth[113,114,330]. Along with selecting high-quality gametes, optimizing the embryo culture system is a complex and multifaceted aspect that also yields a significant role in EQ[306,330,331].

The strategy of embryo *in vitro* culture is to replicate the physiological conditions in the female reproductive tract as closely as possible, to minimize induced stress on embryos[40,114,330]. Over the years, the controlled culture conditions used today have been developed, which aim for constant atmosphere of 5-7% CO_2, 5% O_2, a temperature of 37ºC and a pH of approximately 7.2-7.4[113,330,332,333]. In addition to these parameters, the composition of the culture media is also carefully considered, to ensure that embryo needs are met at each stage of development, either through sequential/two-step media or single step medium[114,330,334]. To achieve these optimal conditions, environmental, physical, chemical and operator related factors must be considered[113,114,335].

To maintain the aforementioned culture conditions stable throughout the embryo development, it is essential to minimize the frequency and duration of observations of gametes and embryos outside the incubator, to avoid fluctuations in temperature and pH[113,114]. This reduction of observations outside the incubator has also been reported to influence the blastulation and proportion of good quality embryos[336]. Additionally, the use of mineral oil overlay on the media in culture dishes provides protection against unfavourable environmental factors, particularly during manipulation outside the incubator, by delaying temperature decreases and preventing media evaporation[40,114,335]. An approach that excludes the need for exposure of the embryos to suboptimal environmental conditions is the use of an incubator with a combined microscope system (time-lapse incubation)[114,125,331].

Another factor that is discussed and can impact embryo development and quality is the use of single or group culture systems[330,331]. It has been reported that grouping the embryos has a positive impact on blastulation and blastocyst quality, with these improvements surpassing the advantages of individual culture[330,337,338].

4.7 Embryo quality

As discussed in the previous section, embryo culture is crucial to guarantee that good-quality embryos are obtained from ART treatments, to ensure high success rates[113,114,330]. It is equally important to accurately assess their quality, allowing for embryo selection from the obtained cohort[202,339]. The selection of the most developmentally competent embryos can be achieved through invasive or non-invasive

criteria[125]. Invasive evaluation can be based on genetic testing for the selection of an euploid embryo, but it is more expensive, time-consuming and carries more risks[340]. Despite its subjective nature, the most commonly employed selection criterion is a non-invasive morphological evaluation of the embryo at different stages of development[204,341,342]. Advantages of this approach relate to a faster and simpler procedure for evaluation, as well as its association with embryo competence and ART outcomes[341,343,344].

A high morphological score has been considered a predictor for live birth[345,346]. Despite reported associations between a good blastocyst morphology and euploidy, a good-quality embryo may not necessarily be associated with the absence of genetic defects, especially in the cleavage stages[343,347]. However, a poor morphology may be indicative of chromosomal abnormality[344], and therefore, it is an adequate criterion for excluding low quality embryos[204], although live births have been reported when low quality embryos have been transferred[348,349].

The effort to standardize embryo morphological evaluation is reflected on the Istanbul Consensus of 2011, that established the specific timepoints for observing embryos during each developmental phase, namely the zygote, cleavage, morula and blastocyst stages[218,340](**Table 9**). It has been reported that correct development within these timings is associated with a higher LBR[225,346].

Table 9: Standard timing of observations for fertilisation assessment and embryo development assessment, with the correspondent expected stage of development for each timepoint, as defined by the Istanbul Consensus of 2011. Adapted from Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011[218]. Hpi- hours post insemination/injection.

	Timing (hpi)	**Expected stage of development**
Fertilisation check	17 ± 1	Pronuclear stage
Day 2	44 ± 1	4-cell stage
Day 3	68 ± 1	8-cell stage
Day 4	92 ± 2	Morula
Day 5	116 ± 2	Blastocyst

It is relevant to evaluate embryo morphology at multiple stages of development[204] (**Fig. 13**), as different criteria are established for each stage regarding EQ and competence[116]. Additionally, the embryo genome is fully activated at the 4-8 cell stage, and prior development is mainly the responsibility of the oocyte[116,127,350]. Hence, embryo competence can only truly be evaluated after this stage[116]. Therefore, a good-quality embryo at cleavage stages may not correspond to a good-quality

blastocyst, and poor-quality cleavage stage embryos may develop into viable blastocysts[351–353].

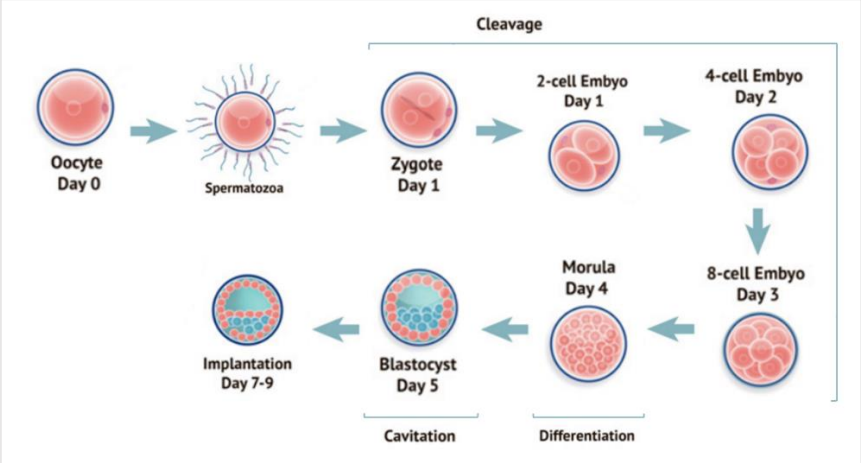


Figure 13: Scheme of early embryo development, from the oocyte to the blastocyst stage. Adapted from Girsh, 2021[40].

One of the scoring systems currently in use was developed by the Embryology Interest Group and the Spanish Association for the Study of Reproductive Biology[341] (ASEBIR). This system, known as the ASEBIR criteria for morphological assessment and selection of early embryos and human blastocysts, includes parameters that have been associated with embryo competency, related to implantation[115,354]. This scoring system will be depicted in the following sections, according to the corresponding stage of development.

The parameters that are usually taken into account for morphological grading of EQ are blastomere number, size, shape and symmetry, fragmentation, compaction, vacuoles and multinucleation[125,204,339]. Several authors report that the most significant morphological parameters for EQ and subsequent reproductive outcomes are cell number and symmetry, as well as fragmentation[40,115,125,129].

4.7.1 Zygotes

In addition to assessing fertilisation on day 1 of development at 17 ± 1 hpi (**Table 9**), simultaneous evaluation of pronuclear morphology can be performed[218,354,355]. Particularly, a non-central positioning of the PN within the cytoplasm (**Fig. 14A**) or a separation between the PN (**Fig. 14B**) may result in subsequent developmental and chromosomal abnormalities[355,356]. A large difference (>4 µm) in PN size (**Fig. 14C**) is also related to developmental and chromosomal abnormalities[218,317]. Therefore, embryos displaying these defects should not be

considered for ET[317,355]. Additionally, cytoplasmatic factors such as the presence of large vacuoles (>14 μm in diameter) (**Fig. 14D**) have also been demonstrated to impair blastocyst development[256]. It is essential to conduct this assessment within the appropriate timeframe (**Table 9**), given the dynamic nature of the PN[115,204,317].

The ASEBIR criteria for morphological assessment and selection of early embryos and human blastocysts do not establish specific grading for day 1 zygotes[354].

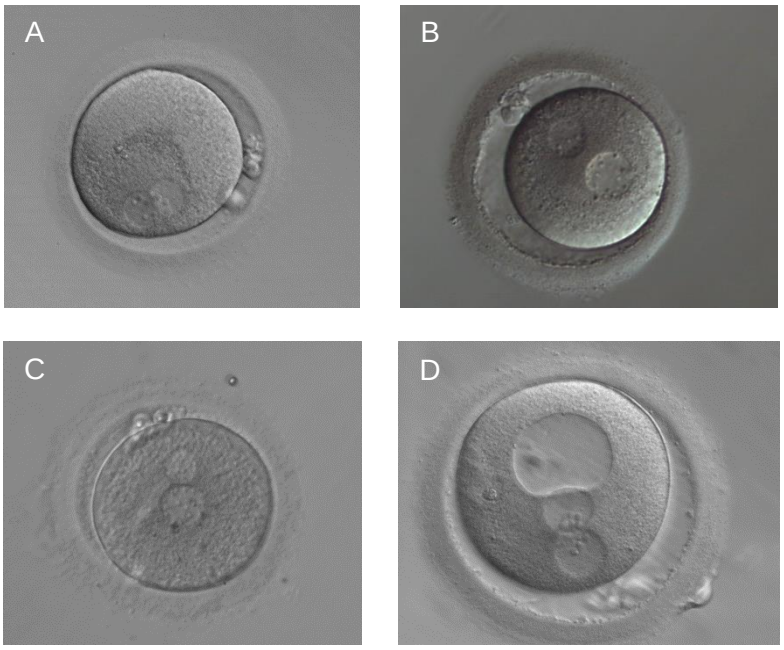


Figure 14: (A) Zygotes with two juxtaposed PNs, in a peripheral position in the cytoplasm. **(B)** Zygote with a wide separation between the two pronuclei, that are slightly different sizes. **(C)** Zygote with two unevenly sized juxtaposed PNs. **(D)** Zygotes with two juxtaposed PNs, in a peripheral position in the cytoplasm, and a large vacuole in the cytoplasm. PN- pronuclei. Adapted from the Atlas of Human Embryology- Chapter 2: The Zygote[317].

4.7.2 Cleavage embryos

The ASEBIR criteria for morphological assessment and selection of cleavage stage embryos (**Table 10**) are characterized by the evaluation of the number of blastomeres, percentage of fragmentation, symmetry of cleavage, presence of multinucleation, and cytoplasmic anomalies[354], at 44 ± 1 and 68 ± 1 hpi, on the second and third day of development, respectively (**Table 9**). The established grades are related to the implantation potential, A being the highest and D the lowest[354].

Table 10: ASEBIR grading system for cleavage stage early embryos. bn- binucleate; NSS- non-stage specific; SS- stage specific; →- from Day 2 to Day 3; *thick, septate, ovoid or dark ZP; ‡with grade A qualities in the other parameters. Adapted from Saiz, et al., 2018[354].

Grade	Day	Cells	Fragmentation	Symmetry	Multinucleation	Others
A	D2	4	≤ 10%	SS	No	Normal
	D3	8				
B	D2	5	> 10-25%	SS	No	Abnormal ZP* or ≤50% cells with small vacuoles
	D3	5 → 7-10 4 → 7, 9, 10				
C	D2	2, 3, 6	> 25-35%	2, 4, 8 cells, NSS	1 bn cell‡	≤50% cells with big vacuoles
	D3	2, 3 → 6-9 6 → 8-10 6, 11, 12			1 or 2 bn cells‡	
D	D2	3 (NSS), >6	> 35%	3 cells, NSS	Other types of multinucleation	>50% cells with vacuoles
	D3	3-5, +1 than D2				

During the cleavage stage, on days 2 and 3 of embryo development at the defined timepoints for observation (**Table 9**), the zygotes should be divided into 4 (**Fig. 16A**) and 8 cells (**Fig. 16B**) respectively[218]. At this stage, the number of blastomeres serves as the primary indicator of quality and competency, as it represents correct cleavage and development[339,354]. Furthermore, day 2 and 3 embryos with the expected number of blastomeres are associated with significantly higher delivery rates and euploidy[357–361]. As such, faster or slower cleavage is associated with a decreased implantation potential[218,360–362]. When embryos exhibit no increase in blastomere number within a 24h period, indicating developmental arrest, they should be discarded due to their low implantation potential, as cell division occurrence is most important indicator of embryo viability[114,218,354,360].

Fragmentation refers to the presence of anucleate cytoplasm fragments of blastomeric origin enclosed by cell membrane, formed during mitosis, that can occur at different degrees[115,354]. It has been reported that embryo development may be impaired and chromosomal integrity affected due to this phenomenon, especially when the degree of fragmentation is high[360–362]. It is measured by percentage of the total cytoplasmic volume[339]. In the ASEBIR criteria, minor fragmentation that has no impact

on embryo implantation potential is defined by ≤10% (**Fig. 16C**), mild is classified as >10-25% (**Fig. 16D**), moderate as >25-35% (**Fig. 15E**), while severe fragmentation is defined by >35%[354] (**Fig. 16F**). Additionally, embryos with >50% of fragmentation (**Fig. 16G**) are characterized by ASEBIR as having a probability of implantation that is close to zero, rendering them non-viable[339,354].

Symmetry of cleavage can be evaluated by considering the blastomere size and numbers[354]. Cell division should result in two blastomeres of equal size, and can happen in a synchronous or asynchronous form[363]. Stage specific (SS) embryos are the ones with even blastomere sizes at 2-, 4- or 8-cell stages[218,354,363] (**Fig. 15**, **16A**, **16B**). In the cases of SS cleavage embryos with 3-, 5-, 6-, 7- or 9-cells, the blastomeres should be uneven-sized, due to the asynchronous cell division[354,363] (**Fig. 15**, **16J**, **16L**). When scenarios other than these are observed, the embryos are considered non-stage specific[363] (NSS) (**Fig. 15**, **16H**, **16I**, **16K**). It has been reported that SS embryos have a significantly higher implantation potential[358,362].

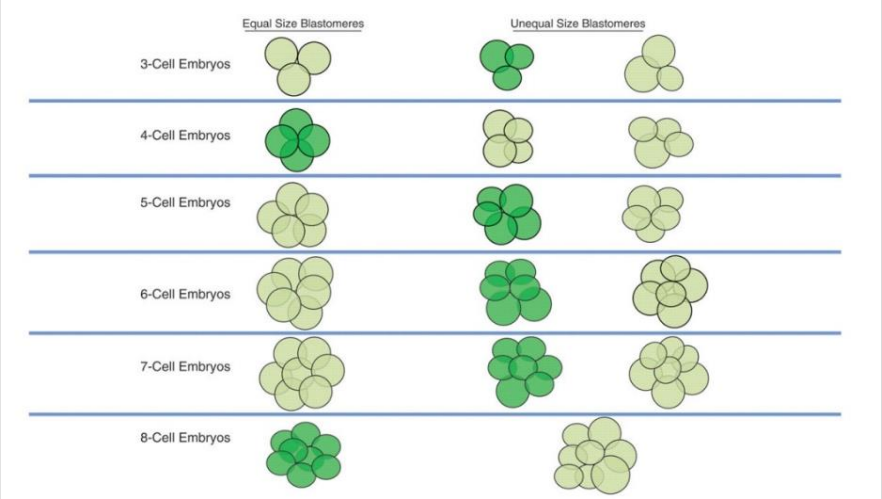
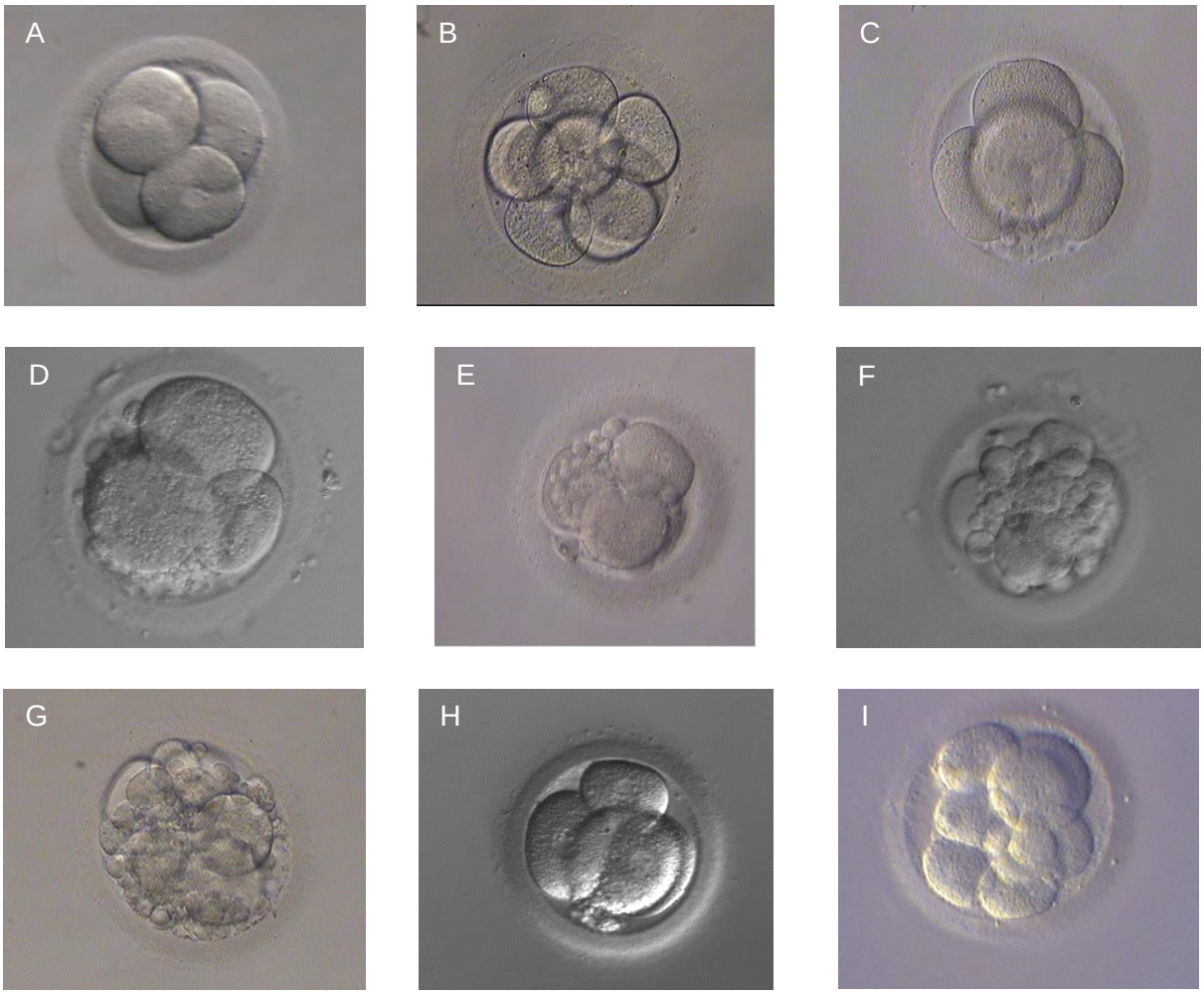


Figure 15: Scheme that distinguishes stage specific (dark green) and non-stage specific (light green) cleavage patterns in day 2 and day 3 early embryos, depending on blastomere size and number. Adapted from Prados, et al., 2012[363].

Multinucleation is present when more than one nucleus is observed in at least one blastomere of a cleavage stage embryo[218,339,364]. The normal situation is for each blastomere to present a single nucleus (**Fig. 16A**), but binucleate (**Fig. 16M**) or multinucleate (**Fig. 16N**) blastomeres can be observed[40,218]. Usually, these abnormal scenarios are more easily detected when a day 2 embryo is assessed (**Fig. 16A**), compared with a day 3 embryo (**Fig. 16B**), due to the presence of more and smaller cells in the latter[204,364]. Multinucleation has been associated with decreased IR, PR and LBR, as well as an increased chance of chromosomal abnormalities[357,359,361,365]. The

presence of multinucleation has been shown to be related with the presence of fragmentation and asymmetric cleavage[364,365].

The cytoplasmic anomalies included in the ASEBIR criteria for morphological assessment and selection of early embryos are the presence of vacuoles at different degrees and zona pellucida abnormalities. The presence of widespread vacuolisation in the embryo may impair embryo development, and embryos with big vacuoles in >50% cells should be discarded due to their low implantation potential[354,363]. Contrarily, the presence of small vacuoles (**Fig. 16O**) does not seem to impact embryo development[218]. Similarly to vacuolization, minor dysmorphisms in the zona pellucida do not significantly impact ART outcomes[354].



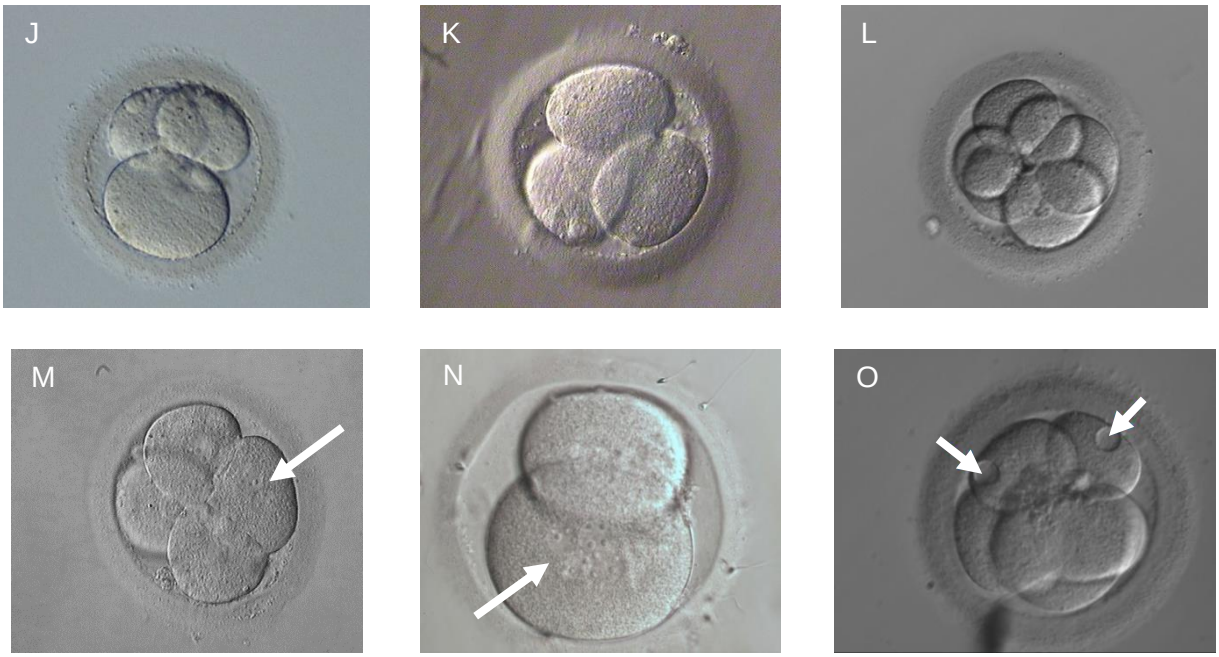


Figure 16: Examples of morphological variations in cleavage stage embryos. **(A)** Day 2 embryo with four evenly sized blastomeres, that display a single nucleus. **(B)** Day 3 embryo with eight evenly sized blastomeres, whose nucleus are not visible. **(C)** 4-cell embryo showing <10% fragmentation. **(D)** 4-cell embryo showing 10-15% fragmentation. **(E)** 4-cell embryo showing 20-25% fragmentation. **(F)** 4-cell embryo showing 40% fragmentation. **(G)** Embryo showing >50% fragmentation. **(H)** Non-stage specific 4-cell embryo with unevenly sized blastomeres. **(I)** Non-stage specific 8-cell embryo with unevenly sized blastomeres. **(J)** Stage specific 3-cell embryo, with unevenly sized blastomeres. **(K)** Non-stage specific 3-cell embryo, with evenly sized blastomeres. **(L)** Stage specific 6-cell embryo, with unevenly sized blastomeres. **(M)** 4-cell embryo with one binucleated blastomere (white arrow). **(N)** 2-cell embryo with four nuclei in one blastomere (white arrow). **(O)** 5-cell embryo with small vacuoles (white arrows) in two blastomeres. Adapted from the Atlas of Human Embryology- Chapter 3: The Cleavage Stage Embryo[339].

4.7.3 Morula

On day 4 of development (92 ± 2 hpi), the embryo should be at the morula stage of development[218] (**Table 9**). The morula corresponds to an 8-32-cell embryo that has either started or completed the compaction stage, in which the boundaries between blastomeres become increasingly indistinct (**Fig. 17A**, **17B**, **17C**), due to an increase in cell-to-cell adherence, resulting in a mass of compacted cells[40,114,218]. A good quality morula is capable of developing into a good quality blastocyst[366].

The ASEBIR criteria for morphological assessment and selection of morula stage embryos (**Table 11**) primarily focuses on the occurrence of compaction and cavitation, but cytoplasmatic characteristics such as fragmentation and vacuolization are also considered. Morphological grading of the embryos at this developmental stage has not been widely studied and applied, as limited information can be obtained due to the lack of individual features[354,367], and due to the need to minimize embryo

manipulation outside the incubator[342]. However, with the emergence of time-lapse incubation, this latter difficulty has been overcome[113].

Table 11: ASEBIR grading system for embryos at the morula stage. Adapted from Saiz, et al., 2018[354].

D3 Grade	Morphological characteristics on D4	D4 Grade
A	Early cavitation Total compaction and >8 cells	A
	Partial compaction (1-2 cells excluded)	B
	Partial compaction (>2 cells excluded) No compaction (>8 cells)	C
B	Early cavitation Total compaction and >8 cells Partial compaction (1-2 cells excluded)	B
	Partial compaction (>2 cells excluded) No compaction (>8 cells)	C
C	Early cavitation Total compaction and >8 cells	
	Partial compaction No compaction (>8 cells)	D
D	>35% fragmentation Widespread vacuoles <50% compacted embryo ≤8 cells without compaction	

An important marker of a good quality morula is the inclusion of all the blastomeres in the compaction process[218] (**Fig. 17D**). When partial compaction occurs due to the exclusion of blastomeres (**Fig. 17E**, **17F**), a loss of blastulation potential has been reported, particularly when more than half of the cells are excluded[368].

When compaction has not started at the timepoint for morula observation (**Fig. 17G**), a negative impact on blastulation is observed compared with embryos that have completed compaction[369,370]. Additionally, a negative impact dependent on the degree of morula fragmentation and vacuolization on blastulation and blastocyst quality has also been reported[362,370]. If cavitation has begun at the timepoint for morula observation (**Fig. 17H**), it is referred to as early cavitation, and it has been associated with better blastocyst quality and ART outcomes[362,371].

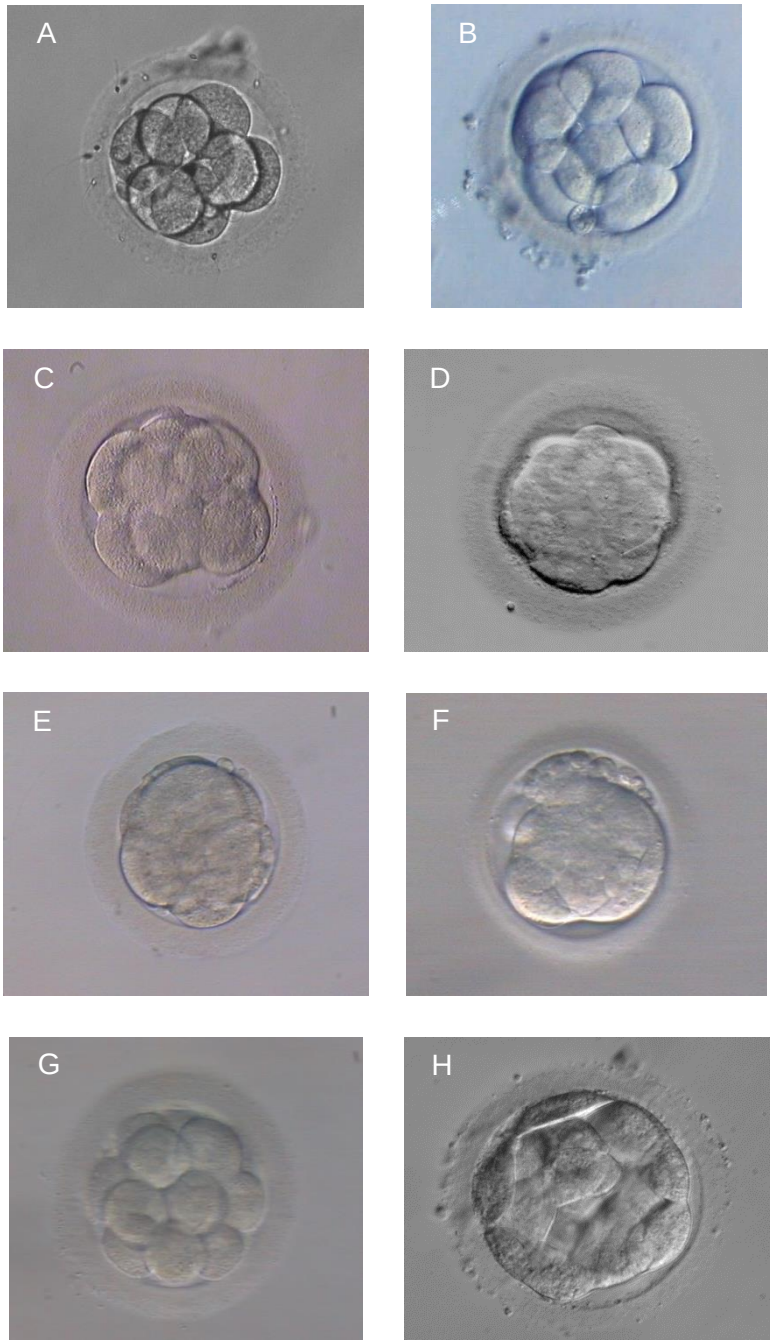


Figure 17: Examples of morphological variations in morula stage embryos. **(A)** 8-cell embryo showing no signs of the start of compaction. **(B)** 8-cell embryo showing signs of initial compaction. **(C)** 8-cell embryo showing signs moderate compaction, with an increased difficulty of distinguishing individual blastomeres. **(D)** Good-quality morula with all blastomeres included in the compaction process. **(E)** Moderate-quality morula, with small cells or fragments not included in compaction. **(F)** Poor-quality morula, with several cells and fragments excluded from compaction. **(G)** Day 4 embryo with more than 12 blastomeres, showing no signs of compaction. **(H)** Day 4 embryo showing early cavitation. Adapted from the Atlas of Human Embryology- Chapter 3: The Cleavage Stage Embryo[339].

4.7.4 Blastocysts

The morphological assessment of a day 5 embryo (116 ± 2 hpi) (**Table 9**) should correspond the observation of a blastocyst stage embryo[218]. A blastocyst is

composed of the trophectoderm (TE), the inner cell mass (ICM) and the blastocoel[372] (**Fig. 18**). The blastocoel is the fluid-filled blastocyst cavity, which forms as a result of the cavitation process[40,114,125].

It has been reported that around 40-50% of cleavage stage embryos reach the blastocyst stage during *in vitro* culture, and that developmental arrest or failure may be caused by chromosomal defects or to suboptimal culture conditions[336,353,373]. According to the Istanbul Consensus, although the morphological selection of blastocysts selects against arrested embryos, which have been reported to occur due to chromosome abnormalities that are not concurrent with blastocyst formation, it does not effectively select against aneuploidy[218,343,361,373,374].

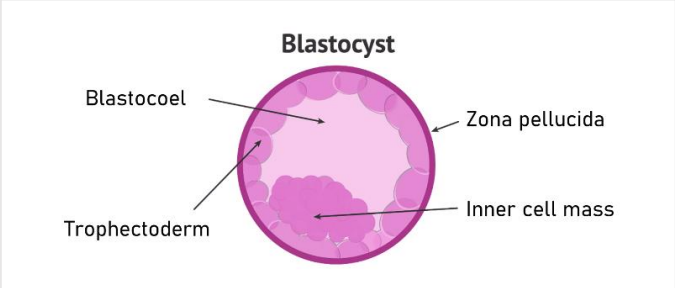


Figure 18: Diagram illustrating the structural organization of a blastocyst. Adapted from Francos Pérez, et al., 2022[367].

The ASEBIR criteria for morphological assessment and selection of blastocysts are defined through the assessment of the degree of expansion of the blastocoel, and the appearance of the TE and ICM. For this assessment, specific criteria were defined for the classification of the ICM and the TE, both focusing on the number and cohesiveness of the cells[354,372] (**Tables 12** and **13**, respectively).

The ICM is located inside the blastocoel, and is composed of a cluster of spherical cells, that will develop into the foetus[114,125]. A good quality ICM is oval, compacted and easily distinguished (**Fig. 19A**), with lower volumes being associated with lower IR[354,375] (**Table 12**). When considering the compaction and number of ICM cells, grade A embryos (**Table 12**) are associated with better ART outcomes compared to lower grade blastocysts, with few loosely bound cells[376,377] (**Fig. 19B**).

The TE cells have a flattened appearance and play an important role in both hatching and implantation, further developing into extra-embryonic structures, such as the placenta[125,378]. According to ASEBIR criteria, a better quality is awarded when the TE cells are homogeneously elliptical, as opposed to a thin and non-cohesive TE with

few cells[354] (**Fig. 19B**) (**Table 13**). Similar to the ICM, grade A embryos regarding TE cells evaluation (**Fig. 19C**) (**Table 13**) are associated with better ART outcomes[376,377].

For both ICM and TE assessment, the presence of degenerated cells (**Fig. 19D**) is a bad prognosis, while the presence of necrotic cells presents as a reason for exclusion from ET[354,379].

Table 12: ASEBIR grading system for ICM of blastocyst stage embryos. ICM- inner cell mass. Adapted from Saiz, et al., 2018[354].

Grade	ICM size (μm^2)	ICM Appearance
A	3800-1900	Compacted
B	3800-1900	Loose
C	1900	Any
D	Ongoing degeneration	
Excluded	Necrotic	

Table 13: ASEBIR grading system for TE of blastocyst stage embryos. TE- trophectoderm. Adapted from Saiz, et al., 2018[354].

Grade	TE description
A	Homogeneous; cohesive; numerous cells
B	Homogeneous; less cells
C	Few cells
D	Ongoing degeneration
Excluded	Necrotic

At the blastocyst stage, the embryo goes through the process of expansion, in which a gradual increase in blastocoel size is observed (**Fig. 19E**, **19F**) due to the accumulation of fluid, resulting in the blastocyst getting progressively bigger, and the zona pellucida progressively thinner[114,125,378]. Eventually, the expansion process leads to hatching (**Fig. 19G**), in which the blastocyst emerges from the zona pellucida, followed by an instantaneous expansion to the fully expanded stage[125,218,378] (**Fig. 19H**).

Regarding the ASEBIR grading, the expansion degree is considered normal from the initial expansion to hatching stages (**Table 14**). It has been reported that a greater degree of blastocyst expansion is related to higher chances of ART success[376,377]. Therefore, when an early blastocyst (**Fig. 19E**) is present at the timepoint of observation on day 5, a worse grade is given. A similar scenario, but with worse prognosis, is encountered when the embryo is still at the morula stage on day 5[354,380].

The ASEBIR criteria for morphological assessment and selection of blastocysts relies on the assumption that the TE grade provides better prognostic value than the ICM grade to attribute the day 5 and day 6 overall grades[354] (**Table 14**). This preference of TE quality over ICM quality for blastocyst selection and as a predictor of ART outcomes has been reported by several authors[377,381]. According to ASEBIR, the morphological grades of the previous days should only be considered for embryo selection when choosing between blastocysts of equal grade[354].

Table 14: ASEBIR grading system for embryos at the blastocyst stage on day 5. ICM- inner cell mass; TE- trophectoderm; ZP- Zona Pellucida. Adapted from Saiz, et al., 2018[354].

Day 4	Morphological characteristics on D5			Day 5
	Expansion degree	ICM	TE	Grade
Morula compacted	From initial expansion to hatching	A	A	A
			B	B
			C	C
			D	D
		B	A	A
			B	B
			C	C
			D	D
		C	A	A
			B	B
			C	C
			D	D
		D	A, B, C or D	D
	Early blastocyst or cavitating (Thick ZP)			C
Morula not compacted	Morula			D

As mentioned before, some embryos go through the developmental process slower than the expected stages. Thus, criteria for evaluation of blastocyst morphology on day 6 of development (138 ±2 hpi) has also been defined by ASEBIR (**Table 15**). However, it has been reported that embryos that reach the blastocyst stage only on day 6 are associated with lower implantation and euploidy rates, which is reflected on the attributed grades[343,382,383].

Table 15: ASEBIR grading system for embryos at the blastocyst stage on day 6. ICM- inner cell mass; TE- trophectoderm; ZP- Zona Pellucida. Adapted from Saiz, et al., 2018[354].

Day 5	Morphological characteristics on D6			Day 6 Grade
	Expansion degree	ICM	TE	
Early blastocyst or cavitating (Thick ZP)	From initial expansion to hatching	A	A	B
			B	
			C	C
			D	D
		B	A	B
			B	
			C	C
			D	D
		C	A	B
			B	
			C	C
			D	D
		D	A, B, C or D	
	Early blastocyst or cavitating (Thick ZP)			
Morula not compacted	Morula			Excluded

The Istanbul consensus established that when a collapsed blastocyst (**Fig. 19I**) is observed, the embryo must be examined again after 1-2 hours, to assess whether the state is maintained, due to the rapidly changing nature of blastocyst morphology[218,379].

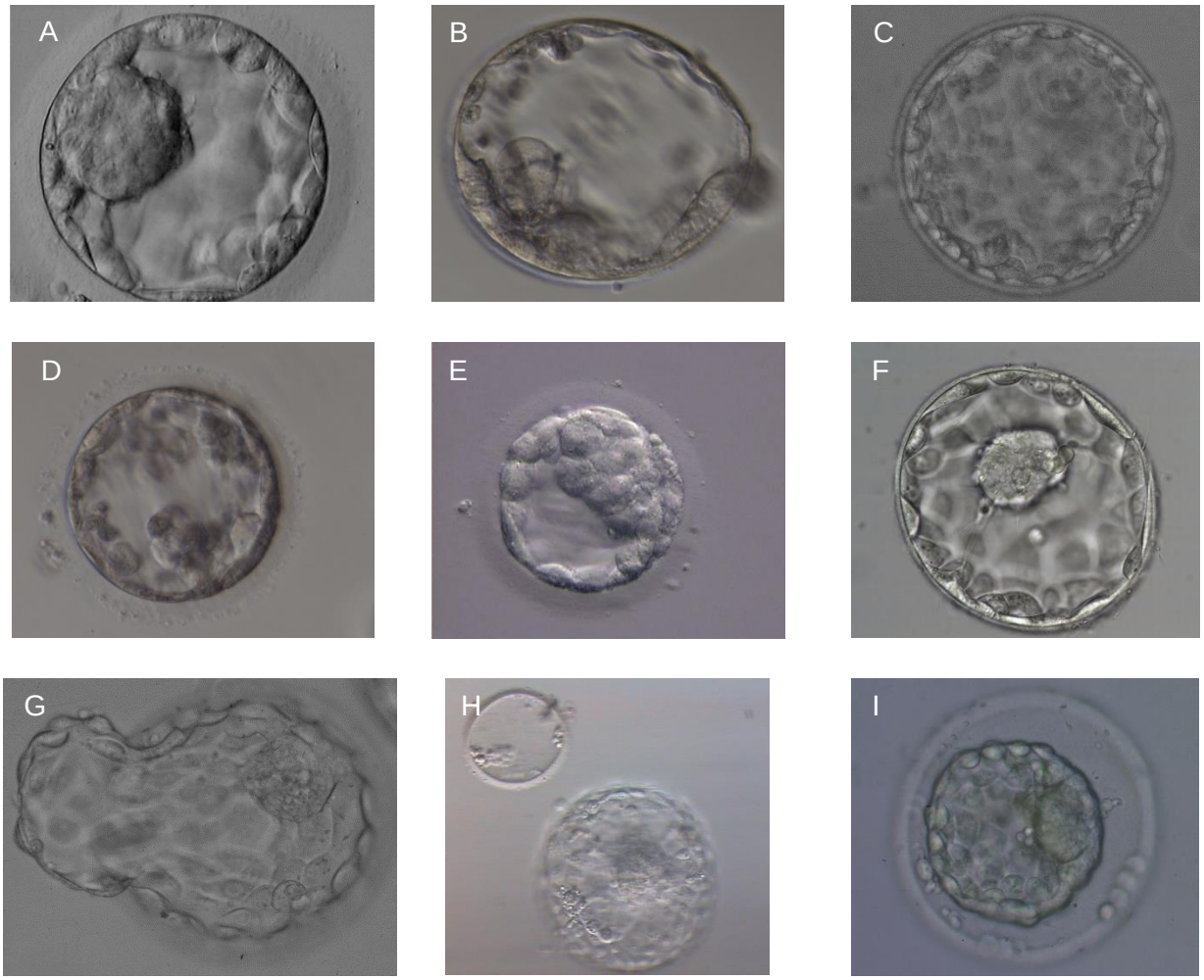


Figure 19: Examples of morphological variations in blastocyst stage embryos. **(A)** Blastocyst with a large ICM, composed of many tightly compacted cells. **(B)** Blastocyst with a loose ICM and a non-cohesive TE, composed of few cells. **(C)** Blastocyst with a cohesive TE, composed of many cells. **(D)** Early poor-quality blastocyst, with degenerated cells both in the ICM and TE. **(E)** Early blastocyst, with the blastocoel cavity making up about half of the embryo, still with no thinning of the ZP. **(F)** Expanded blastocyst, showing an increase in embryo volume and a thinned ZP. **(G)** Blastocyst in the process of hatching. **(H)** Hatched and fully expanded blastocyst, and the correspondent empty zona pellucida. **(I)** Collapsed blastocyst within the zona pellucida. ICM- inner cell mass; TE- trophectoderm; ZP- zona pellucida. Adapted from the Atlas of Human Embryology- Chapter 4: The Blastocyst[372].

4.8 Embryo transfer

The ET is a simple procedure, that is the ultimate point of an ART cycle[125,384]. It relies on the loading of a catheter (**Fig. 20**) with the embryo(s), and their transfer into the uterine cavity[125]. The success of the ET does not depend only on EQ, but also on transfer technique and endometrial receptivity[384].

For the loading of the catheter, it is important to minimize the volume of culture medium that is aspirated with the embryo(s) (15-20 µL), to avoid expulsion or dislocation of the embryo(s) after ET[113,204,278,384]. One of the methods that can be used for the loading of the catheter is the medium-air-embryo-air-medium technique, in

which two air bubbles are aspirated, before and after the medium that contains the embryo(s)[40,384] (**Fig. 20**). This method enables the visualization of the embryo's location within the catheter and the uterine cavity post-transfer, through transabdominal ultrasound imaging[40,125]. Additionally, it is also important to minimize the time between catheter loading and embryo release into the uterus, to protect the embryo from environmental stress as much as possible[40,113,384].

After the ET, it is essential that the catheter is observed, to verify that the embryos are not retained, and have been successfully transferred[113]. If that is not the case, the embryos should be loaded into a new catheter, and ET should be repeated[204]. The retention of the embryo(s) in the catheter and subsequent ET attempt have been shown to not impact the outcomes of the cycle[385,386].

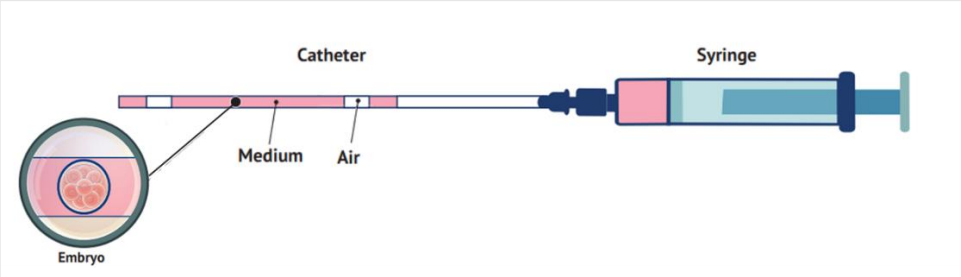


Figure 20: Diagram representing the medium-air-embryo-air-medium catheter loading technique. The total volume of medium in the catheter should not exceed 30 µL, with 15-20 µL in the embryo-containing portion of medium[113,384]. Adapted from Girsh, 2021[40].

As mentioned in previous sections, the embryo selection is performed through morphological criteria. There are additional aspects that should be considered prior to the procedure, namely the number and developmental stage of the embryo(s) that will be transferred.

The decision regarding the number of embryos to transfer is primarily based on maternal age, the number of good quality embryos, and their respective developmental stage[40,116,387]. Although the transfer of more than one embryo raises the likelihood of implantation, it also increases the possibility of multiple gestation, which has been correlated with obstetrical and neonatal complications[125,388].

Therefore, to decrease the incidence of multiple gestation in ART, it is highly recommended to proceed with elective single embryo transfer (eSET) when possible, while the remaining good quality embryos are cryopreserved[55,127,388]. It has been reported that it is possible to decrease the multiple pregnancy rate while still maintaining similar clinical PR when eSET is conditionally applied[389–391]. The importance of a successful cryopreservation and warming procedure has been

highlighted to guarantee that the frozen embryos will positively contribute to the cumulative rates[113,392].

The patients for which eSET is recommended are those with a favourable prognosis, i.e., young maternal age (<35 years), undergoing the first two ART cycles, having more than one good quality embryo, or using donated oocytes[125,204,392]. Contrarily, for women older than 37 years old, with poor EQ, or undergoing the third ART cycle, the transfer of two embryos might be considered[352,387].

It has been reported that the clinical pregnancy and LBR is superior when a blastocyst is transferred, compared to the transfer of a cleavage stage embryo[393,394], with higher multiple gestation rates when two blastocysts are transferred[395]. Therefore, when a good quality blastocyst is available, eSET is highly advised[387,392].

The main advantage of transferring blastocysts rather than cleavage stage embryos is that it allows for selection after embryonic genome activation, and in the case of fresh ET, a more adequate synchronicity with the endometrium[125,204,350]. However, leaving the transfer for day 5 of development comes with the increased risk that no embryos will reach the blastocyst stage, and ET will have to be cancelled[40,393]. For the same reason, the number of frozen embryos is also reduced when day 5 transfer cycles are considered[393,394]. Usually, the decision of whether to perform a cleavage or blastocyst stage transfer is contingent on the number of available embryos, i.e., when few embryos are viable on day 3, cleavage stage transfer may be considered, to preclude ET cancellation on day 5[40,113,125].

To confirm biochemical pregnancy, a blood test for ß-hCG (human chorionic gonadotropin) serum levels should be done 11 or 9 days after the ET, for cleavage or blastocyst stage transfers, respectively[4,116]. Clinical pregnancy is confirmed 14 days later, by the ultrasonographic visualization of a gestational sac with foetal heartbeat[4].

4.9 Cryopreservation

Cryopreservation is the process that enables the preservation and storage of oocytes, sperm and embryos, and is considered a key component of ART[40,116]. The cells are stored in liquid nitrogen tanks at a temperature of -196ºC[114,115].

The cryopreservation of human gametes is performed to preserve the reproductive potential in various situations, such as prior to clinical treatments that may cause the loss of reproductive function (e.g., radiotherapy, chemotherapy, certain surgical interventions), before hormonal therapy in transsexualism cases, for donor

programs or for social preservation[40,113–115]. Additionally, gametes can be cryopreserved when surgical removal of sperm is necessary, or when sperm collection was not possible after successful OPU[40,114].

As for the cryopreservation of embryos, it is routinely performed for remaining good quality embryos after fresh ET or when the fresh ET is not possible or advisable[113,114,125]. Cryopreservation allows for subsequent ETs to occur without the need for OPU[55]. Additionally, as mentioned in the previous section, it is an essential safeguard to permit the rise of eSET and subsequent decrease of multiple gestation rate[278].

Cryopreservation can be carried out using different methodologies, such as slow freezing and vitrification[114]. Slow freezing has been replaced by vitrification as the primary cryopreservation method for oocytes and embryos[116,125,278].

The goal of cryopreservation is to have minimal impact on embryo viability and ensure survival after thawing[127]. The main risk associated with cryopreservation is the formation of intracellular ice crystals[113,115]. Other risks include osmotic and cold shock[114]. Cryoprotectants are used in the freezing procedure to prevent osmotic stress and the formation of ice crystals, by slowly replacing the intracellular water[113–115]. The exposure of the cells to increasingly higher solutions of cryoprotectants results in the dehydration of the cell and subsequent shrinkage and recovery[40,115,129]. Cryoprotectants can be cytotoxic towards cells, highlighting the importance of reducing exposure times in optimized protocols[116,129].

Contrarily to oocytes and embryos, sperm cryopreservation is most commonly performed through the rapid-cooling method[115]. This procedure involves slowly mixing non-processed or processed sperm with an equal volume of cryoprotectant, that is then aliquoted into straws or cryovials and exposed to liquid nitrogen vapour before being stored in liquid nitrogen tanks[40,114,129].

4.9.1 Vitrification

Vitrification involves rapid cooling rates and exposure to high concentrations of cryoprotectants, in contrast to the slow freezing method[113,115]. The exposure to hypertonic conditions results in fast dehydration, and the rapid cooling rates are achieved by keeping the cells in minimal volumes of media, that are then loaded onto carrier devices and exposed to and stored in liquid nitrogen[40,114–116].

These characteristics result in the vitreous state of intracellular water, avoiding the formation of ice crystals, and thus minimizing damage to cellular structures[114,115]. Compared to slow freezing, vitrification requires higher levels of training, but is less time-consuming[114,129] and is associated with increased survival rates of both oocytes and embryos[396,397]. Vitrification can be implemented at all stages of embryo development, but is more commonly carried out at the cleavage or blastocyst stages[40,116].

4.9.2 Warming

The warming process involves the rehydration of the cryopreserved cells[115]. This is achieved through exposure to stepwise dilution solutions of an osmotic buffer at 37ºC to ensure gradual removal of cryoprotectants from the cell while preventing osmotic shock[40,113,114]. The process of cryoprotectant removal and water entry is expected to be accompanied by cell shrinkage/collapse and re-expansion[40].

The survival of an embryo after warming is assessed by morphological evaluation and is defined by the presence of >50% of intact blastomeres and/or resumption of mitosis[40,114,127]. The latter is applied when cleavage stage embryos are cultured until the blastocyst stage post-warming[114]. Embryos should be incubated for 4-6 hours before transfer after warming[40]. Oocytes should be cryopreserved immediately after denudation, which should take place 3-4 hours after OPU, corresponding to 38–40 hours post-trigger[40,113]. After warming, oocytes should be incubated for 1-3 hours, so that ICSI is performed at the equivalent of 40–41 hours post-trigger[40].

For semen samples, the warming process is simpler, entailing incubation at room temperature or exposure to 37ºC, depending on the commercial kit used[115,116]. After warming, cryoprotectant should be removed from the sample, by washing, SU or DGC procedure, contingent on the quality of the sample and the steps done prior to freezing[114,129].

Chapter 2 – Intrauterine insemination success: Analysis of influential parameters

1. Introduction

The intrauterine insemination (IUI) procedure was the first available treatment for infertility, introduced in 1962[40,129]. This procedure warrants a higher concentration of viable motile sperm in the uterine cavity around the time of ovulation, through sperm washing and processing before insemination[40,116] (section 4.2, Chapter 1). However, the clinical pregnancy and live birth rates of IUI with autologous sperm are reported to be around 12% and 8%, respectively[271,272].

Despite its lower success rate, IUI is widely employed as a first-line treatment for infertility, due to its simplicity and cost-effectiveness[116,129,263]. Some authors stand for its use as a pre-IVF treatment if prerequisites are met, where three to six IUI cycles should be performed prior to embarking on *in vitro* fertilisation (IVF) cycles[129,398,399]. Prerequisites include at least one non-blocked Fallopian tube and normozoospermia or mild male factor (enough to enable the attainment of ≥1-3 million motile sperm cells after laboratory processing)[129,265].

However, there is a need for an improvement of the IUI procedure, in order to optimise clinical pregnancy and live birth outcomes[263,265]. IUI is a more natural procedure compared to IVF and intracytoplasmic sperm injection (ICSI), and therefore, it would be advantageous if the success rate was higher[40,263].

The optimisation of the IUI treatment has been mainly focused on identifying predictors of success, among various parameters that can be considered in the procedure[400]. The rationale behind is that if the predictors or contributors for IUI success are identified, there would be more room for individual optimisation in each IUI cycle[263,265].

Numerous factors contribute for IUI success, which can be broadly categorized into patient-specific characteristics (age; body mass index (BMI); lifestyle and environmental stressors; type, duration and aetiology of infertility) and cycle-related characteristics (number of attempts; ovarian stimulation and ovulation triggering protocols; clinical monitoring; sperm sample parameters; insemination procedure). The influence of these and other features has been widely studied, although it is difficult to

isolate each individual impact. Moreover, it is important to examine their impact as a set of factors, given their collaborative role in determining the success or failure of the cycle[267].

It has been reported that female age and inseminated sperm count are the factors that have a more relevant impact on IUI success[40,116,399]. It has been consistently reported that increasing female age has a negative impact on clinical pregnancy (CP) after IUI, especially in women older than 40 years old[398,401–404]. Contrarily, sperm count after processing (number of inseminated sperm) is not consistently reported as a significant factor for CP after IUI[270,398,405]. Although there is a recorded association between post-wash total motile sperm count and clinical pregnancy rates, a clear cut-off of sperm count for IUI success is not defined[399,401,405–408]. Several thresholds have been supported, such as 1, 2, 5 and 10 million post-wash total motile sperm cells, but more studies are necessary to clearly define the role of this parameter as a predictor for CP following IUI[406,408–410].

Another sperm parameter that is studied for its influence on CP after IUI is sperm morphology. This parameter is not consensual as a predictor for IUI outcome, when the strict criteria is used for assessment[403,411–413]. It has been suggested that isolated teratozoospermia should not be considered as an exclusion criterion for IUI, but as a co-factor along with other parameters[412,414]. This viewpoint has been supported by findings that CP following IUI is not significantly different in cases with low (<5%) morphology and normal remaining parameters[414], and that sperm morphology gains predictive power in cases with $<1x10^6$ post-wash total motile sperm cells[167].

As mentioned before, other cycle characteristics can influence the outcome of IUI. Regarding the number of IUI cycle attempts, it has been reported that most pregnancies from IUI cycles are derived from the first three to four cycles that patients go through[402,411]. In addition, the stimulation regime may have a substantial impact on IUI outcomes[60,267,270]. The number of follicles is also a relevant factor for IUI success, as it has been reported that pregnancy rates are significantly increased in bifollicular and multifollicular cycles, compared to monofollicular ones[268,270]. However, there is also a confirmed positive association between follicle number and the odds of multiple gestation[268], which is connected with obstetrical and neonatal complications[125,388], therefore justifying the cancellation of cycles with more than two developing follicles[265,267]. Pertaining to endometrial thickness, it is a measure that represents endometrial growth commanded by the hormonal stimulation and is employed as a marker of endometrial receptivity[415]. While women in IUI cycles leading to CP have

been found to present a significantly thicker endometrial lining than in cycles with no pregnancy[416,417], that association is not consistently reported as a significant factor in multivariate analysis[418,419]. Finally, it is essential that the period from ovulation trigger to IUI is adequate (32-36h), thereby facilitating *in vivo* fertilisation[420]. Nonetheless, there are a number of studies reporting no significant differences in IUI outcomes when the procedure was carried out at various timepoints after ovulation triggering[421–423].

Regarding patient-specific features that may influence IUI outcomes, male age does not exhibit a direct significant impact on pregnancy following IUI as female age does[411,424], but it may exert indirect influence through the associated increase of sperm DNA fragmentation, that significantly affects clinical pregnancy rates[72,181]. Female BMI has been shown to not significantly impact the pregnancy rates (PR) following IUI[401,404], even though it has been reported to yield a positive association with both hormonal stimulation dosage and endometrial thickness, and a negative one with number of follicles[425,426]. Similarly, male BMI does not seem to be a significant factor for IUI cycle success[427], despite its connection with reduced semen quality[98,100]. These associations emphasize the importance of ensuring an adequate ovarian stimulation protocol and a semen processing method to sustain IUI pregnancy rates across varying BMI categories. Nevertheless, the existing literature is deficient in addressing this matter, warranting further studies.

One of the lifestyle habits whose influence has been studied on IUI outcomes is the smoking patterns of both the female patient and the male partner. Most studies focus on the occurrence of smoking or not, with contradictory findings on whether a significant difference exists in clinical PR between female smokers and non-smokers[398,428,429]. However, there is an advantage of studying this variable as categorical, as it enables for a more tailored implementation for distinct patients. When the smoking habits are divided into categories, Thijssen et al. reported that male smoking habits have a significant impact on the odds of clinical pregnancy (1-14 cigarettes/day with non-smoker as the reference), but the same was not stated for female smoking[430]. Huyghe et al. reported a significant impact on the odds of clinical pregnancy for female smoking habits in cycles with donor semen (non-smoker and 1-14 cigarettes/day with ≥15 cigarettes/day as the reference) and for male smoking habits (1-14 cigarettes/day with non-smoker as the reference)[431]. The inconsistency of the available data hampers the suitability of this parameter as a predictor for IUI success, although it has been suggested that the impact of smoking should be studied on the obstetrical and perinatal outcomes[398,399].

IUI is indicated as an appropriate treatment for several infertility aetiologies, such as ovulatory dysfunction, unexplained infertility, mild male factor, cervical factor, mild endometriosis, partial tubal factor and ejaculatory/coital factors, but contraindicated in cases of severe endometriosis, bilateral tubal factor or severe male factor[125,264,267,420]. It has been established that IUI success varies significantly between aetiologies, with ovulatory dysfunction often being pointed as the aetiology associated with higher clinical PR[409,432,433]. In the case of endometriosis, it has been reported that PR significantly differs between cases of stages I-II and stages III-IV, with the latter being associated with lower PR[434]. Although some report that unexplained infertility is associated with higher odds of pregnancy than male or female factors, others report the opposite[405,411,435]. Male subfertility as an independent indicator for IUI treatment is not a consensual matter[436], especially due to the absence of strict definitions for mild and severe male infertility, which leads to each study using their own criteria[437] and interferes with the ability to draw sustained conclusions[267]. Both the type and duration of infertility have contradictory reported results on their significance for the clinical PR following IUI[270,405,416,428]. However, both parameters have demonstrated significance in that rate when comparing women under 30 or 35 years old with their older counterparts[409,438].

The present study of the influence of these potential factors on clinical pregnancy following the IUI procedure will be performed through a binomial logistic regression model. This model allows for the simultaneous analysis of the impact of various independent variables on the binary outcome variable[439]. Furthermore, a backward elimination process will be employed, to attain a final model that is less complex, easier to interpret and that presents a better fit to the data[440]. Backward elimination is started with a full model (all the independent variables are included), and is based on the iterative removal of the least significant independent variable, until the most parsimonious model is achieved, by testing its significance at each stage of elimination[441,442].

The aim of this retrospective analysis was to assess the impact of a set of patient and cycle factors on the likelihood of clinical pregnancy following first IUI attempt, on a dataset spanning 10 years of data from IUI cycles, through binomial logistic modelling.

2. Methods

2.1 Data collection

The dataset employed for the present statistical analysis originates from first IUI cycle attempts carried out in the *Unidade de Medicina da Reprodução* (UMR) at *Hospital de Santa Maria* (HSM), between 2011 and 2021. This temporal span was defined from the initial moment of available data until the moment at which clinical pregnancy information was available at the time of the start of the study. To ensure that independence of observations was present and avoid errors in estimation[439], exclusively the first IUI cycle of each couple was included in the dataset.

Cycles of donor or frozen sperm were excluded from analysis to minimize potential confounding factors, given that the freeze-thaw procedure may adversely impact quantity and quality of motile viable sperm[443,444]. Only cases in which the female partner was ≤40 years old were included in the dataset, due to inclusion criteria for the different ART treatments in the UMR.

Additionally, cases of natural IUI cycles (absence of ovarian stimulation and ovulation induction) were excluded, ensuring homogeneity in procedural characteristics across all encompassed IUI cycles. Cases with missing data in some of the evaluated parameters were defined as missing values.

2.2 Intrauterine insemination procedure

The IUI cycles began with ovarian stimulation with the objective of eliciting the development of 1-2 follicles within the ovaries. The initial dosage of gonadotrophin (50-75 IU/day) was contingent on the patients' characteristics. It was essential to monitor the growth of the developing follicles by transvaginal ultrasound, to count the number of developing follicles and measure their diameter. Adjustments to the stimulation regimen were implemented if necessary, according to the established protocol. The administration of ovulation-triggering agents (Ovitrelle 250 or Pregnyl 5000, Merck, Rahway, New Jersey, U.S.) took place 24-36 hours prior to the insemination procedure, upon the observation of either one follicle with a diameter of ≥18 mm or fewer than three follicles with a diameter of ≥15 mm.

All couples were asked to comply with a period of 2-5 days of sexual abstinence prior to treatment. On the day of the IUI procedure, semen samples were collected through masturbation into a wide necked, sterile, disposable, non-toxic for

spermatozoa, room temperature, and identified container[112]. The samples were analysed, and their volume, viscosity, motility, concentration and morphology assessed (section 2.2, chapter 1). Subsequent processing of the semen was performed with washing and swim-up, employing G-IVF™ PLUS culture medium (Vitrolife, Gothenburg, Sweden) as the migration medium (section 2.3.1.1, chapter 1). Preceding the insemination, 0.2-0.5 mL of the processed sample were loaded into a catheter, following which the IUI procedure was conducted, entailing the deposition of the sample within the uterine cavity. Luteal phase support (300 mg/day) was started two days after the procedure, and continued until the three week-mark, at which a pregnancy test was taken. If the test yielded a positive outcome, the luteal phase support was continued, and serum ß-hCG levels were tested. At the 6th-8th week of gestation, a transvaginal ultrasound was performed, to confirm a gestational sac with foetal heartbeat.

2.3 Variables

The outcome variable, defined as the binary dependent variable on the binomial regression model, is the occurrence of a clinical pregnancy. A clinical pregnancy was defined by the presence of a gestational sac with foetal heartbeat at 6-8 weeks after the IUI.

The initial predictor variables, numerical and categorical, encompass both patient and cycle characteristics:

- **Female age**: Age of the female partner undergoing IUI;
- **Female BMI**: BMI of the female partner undergoing IUI;
- **Female smoking habits**: Smoking habits of the female partner undergoing IUI, categorised as Non-smoker, Past smoker, <10 cigarettes/day, 10-20 cigarettes/day or >20 cigarettes/day;
- **Male age**: Age of the male partner;
- **Male BMI**: BMI of the male partner;
- **Male smoking habits**: Smoking habits of the male partner, categorised as Non-smoker, Past smoker, <10 cigarettes/day, 10-20 cigarettes/day or >20 cigarettes/day;
- **Infertility duration**: Length of time, in months, the couple has dealt with infertility;
- **Type of infertility**: Type of infertility experienced by the couple, that takes the value 1 for primary infertility, or the value 2 for secondary infertility;

- **Infertility diagnosis**: Infertility aetiology diagnosed in each case, categorised as Unexplained for unexplained infertility, Male factor for mild or moderate male factor infertility, Partial tubal factor for cases of tubal female infertility where one of the Fallopian tubes is blocked or damaged, Endometriosis for all stages of endometriosis, Ovulatory factor for anovulation and other ovarian factors infertility cases, or Mixed for male and female factors infertility cases;
- **Stimulation total dosage**: Total dosage of follicle-stimulating hormone administered for ovarian stimulation;
- **Mature follicles**: Number of mature follicles, defined by a diameter of ≥16 mm, measured by ultrasound visualisation;
- **Endometrial thickness**: Thickness of the endometrial lining, in mm, measured by ultrasound visualisation;
- **Time interval from trigger to IUI**: Duration, in hours, of the time interval between the administration of the ovulation trigger and the IUI procedure;
- **Inseminated sperm count**: Millions of total motile sperm inseminated in the IUI procedure;
- **Normal morphology sperm**: Percentage of sperm with normal morphology, according to the third edition of the World Health Organisation laboratory manual[445] for the examination and processing of human semen.

2.4 Statistical analysis

IBM-SPSS Statistics version 29.0 for Windows (IBM Corp., Armonk, N.Y., USA) was used for the statistical analysis.

2.4.1 Descriptive statistics

The characteristics of the entire cohort are presented as mean ± standard deviation (SD) for numerical variables, or as a count and corresponding percentages for categorical variables.

2.4.2 Variable pre-selection

There was a need to decrease the number of total variables in the model, to decrease its complexity and facilitate its interpretation, while maintaining the statistical power of the model[440]. Therefore, a preliminary selection of the variables was carried out[440], based on an initial exploratory analysis:

T-tests were performed for the numerical variables, comparing the means of each variable between the cycles that resulted in pregnancy and the cycles that did not. Prior to the t-tests, normality of the numerical variables was tested with the Shapiro-Wilk test, and homogeneity of variances was checked during the tests.

For categorical variables, chi-square tests were performed, comparing the distribution of the categories between the two pregnancy outcome groups. The percentage of cells with counts of less than 5 in the cross-tabulation was kept at less than 20%[446].

Variables that exhibited a high p-value (>0.9) were considered to lack enough statistical significance to the association with clinical pregnancy occurrence, and were excluded from further analysis[441]. This approach allowed for initial exclusion of variables that have a weaker relevancy for clinical pregnancy from the binomial logistic regression, ensuring a less complex model, with easier interpretation and higher validity[440].

Additionally, the strength and direction of potential bivariate correlations between pairs of numerical independent variables were tested through the non-parametric Spearman coefficient. Values of the coefficient higher than 0.7 were considered to demonstrate a strong correlation between two variables[447]. This allows to identify redundancies in the data, present when two numerical variables explain similar variability in the dataset.

2.4.3 Binomial logistic regression model with backwards elimination

A binomial logistic regression model was used to examine the relationship between the chosen independent variables and the likelihood of success of the binary outcome variable[439], clinical pregnancy after first IUI attempt.

The outcome of the logistic regression showcases the influence of the variables on the log-odds of the probability of success[439] through regression coefficients (β)[448] for each independent variable. The regression coefficients are calculated based on the observed data from the cohort, and represent the variation in log-odds with a one-unit change in the value of the correspondent variable, when the other variables are kept constant[449]. The intercept term, also known as the constant, corresponds to the log-odds of clinical pregnancy when all independent variables are set to zero or are at their reference levels[449,450].

The coefficients can then be expressed as Odds Ratios (OR), that are obtained by exp(β)[449]. When the OR value is superior to 1 (β>0), it indicates that the odds of success improve with a one-unit increase in a numerical continuous variable value, while a OR of <1 (β<0) means that a one-unit increase in the variable value will decrease the odds of success[448,449]. A p-value <0.05 was considered statistically significant, and a p-value 0.05 < p < 0.1 was considered marginally significant[451]. A p-value <0.05 showcases that the corresponding variable is a significant contributor to the model[450].

Through the process of backward elimination, a final model that includes the independent variables that contribute to the variation of the observed data by the model was obtained. In this analysis, the most parsimonious model was chosen based on the Omnibus test of model coefficients. This test focuses on the significance of the variable coefficients in the model at each step in the backwards elimination process compared to the null model (with no independent variables)[449]. Therefore, a model displaying a p-value<0.05 is statistically different from the null model, meaning that the included independent variables impact the dependent variable's variation[449]. The first iteration model with a non-significant p-value (>0.05) was not considered more parsimonious than the previous one, which would be the final model.

The overall fit of the final model was assessed with the Hosmer-Lemeshow test for goodness-of-fit and the Nagelkerke pseudo-R^2. The Hosmer-Lemeshow test evaluates if there is a significant difference between the observed and model-predicted outcomes' frequencies[450]. A good fit between the model and the data is present when a non-significant result (p-value>0.05) is obtained[450]. The Nagelkerke pseudo-R^2 was computed to estimate the proportion of variance of the log-odds of the dependent variable that is predicted by the independent variables included in the model[442]. The obtained pseudo-R^2 can vary from 0 to 1 and a higher value dictates a better fit of the model to the data[442].

Results of the final model obtained by binomial logistic regression with backwards elimination analysis were expressed as β, OR and its 95% confidence interval (CI).

3. Results

3.1 Descriptive statistics

A total of 1077 IUI cycles performed at UMR in HSM between 2011 and 2021 were included in the analysis, with all of these cycles representing first IUI attempts. The clinical pregnancy rate was 12.5% (135/1077); live birth rate was 9.7% (105/1077); and within the clinical pregnancy cases, there were 28/133 miscarriages (2 cases with missing data on live birth outcome).

The descriptive statistics for patient and cycle characteristics of the whole study population are summarized in **Table 16.** The mean female age was 34.39 ± 3.86 years (19-40; median: 35). The mean male age was 35.84 ± 5.08 years (22-57; median: 36). The most common infertility aetiology was unexplained infertility (68.7%), and the majority of IUI cycles were performed on primary infertility couples (Type of infertility: mean 1.31 ± 0.46; 1-2; median: 1). The mean total dosage of gonadotrophin for ovarian stimulation was 530.65 IU ± 261.23 IU (150-2775; median: 450), and in the majority of cycles, a single follicle was present at the ovulation trigger (Mature follicles: mean 1.44 ± 0.61; 1-4; median: 1). The post-wash sperm samples presented a mean of 10.66 ± 10.13 million sperm cells (0.010-48; median: 7.2) and a mean of 35.04% ± 9.83% normal morphology sperm cells (1-62; median: 35).

Table 16: Descriptive statistics of patient and cycle characteristics of the entire cohort. *≤1% missing values; ‡≈25% missing values. SD- standard deviation.

		N (%) or Mean ± SD
Patient Characteristics (N= 1077)		
Female age (years)		34.39 ± 3.86
Female BMI (kg/m2)		24.19 ± 4.47
Female smoking habits*		
	Non-smoker	768 (71.9%)
	Past smoker	84 (7.9%)
	<10/day	125 (11.7%)
	10-20/day	71 (6.6%)
	>20/day	20 (1.9%)
Male age (years)		35.84 ± 5.08
Male BMI‡ (kg/m2)		26.36 ± 3.57
Male smoking habits‡		
	Non-smoker	458 (57.3%)
	Past smoker	94 (11.8%)
	<10/day	93 (11.6%)
	10-20/day	112 (14.0%)
	>20/day	42 (5.3%)
Infertility duration* (months)		40.96 ± 21.66
Type of infertility*		1.31 ± 0.46
Infertility diagnosis*		
	Unexplained	738 (68.7%)
	Male factor	192 (17.9%)
	Partial tubal factor	75 (7.0%)
	Endometriosis	54 (5.0%)
	Ovulatory factor	9 (0.8%)
	Mixed	6 (0.6%)
Cycle characteristics (N= 1077)		
Stimulation total dosage* (IU)		530.65 ± 261.23
Mature follicles*		1.44 ± 0.61
Endometrial thickness* (mm)		9.80 ± 1.95
Period from trigger to IUI* (hours)		35.05 ± 3.43
Inseminated sperm count (million)		10.66 ± 10.13
Normal morphology sperm* (%)		35.04 ± 9.83

3.2 Variable pre-selection

The preliminary selection of the variables resulted in the exclusion of the Female BMI and Stimulation total dosage variables, considering univariate analysis results, since no strong correlation was found between variables (**Table A1** and **Table A2**). The remaining subset of variables were considered to have a potential impact on clinical pregnancy after IUI. Additionally, two variables had to be excluded from the binomial regression analysis, due to a high percentage (≈25%) of missing values: the Male BMI and Male smoking habits parameters were not registered during 2011, 2012 and part of 2013, with a total of 278 cycles with those missing values.

Therefore, the variable set that was included on the initial binomial logistic regression model was:

Female age	Infertility duration	Mature follicles
Female smoking habits	Type of infertility	Endometrial thickness
Male age	Infertility diagnosis	Inseminated sperm count
	Period from trigger to IUI	Normal morphology sperm

3.3 Binomial logistic regression model with backwards elimination

Due to missing data on at least one variable, 27 cases were excluded from analysis, resulting in a total of 1050 cases being included.

The binomial logistic regression with backwards elimination analysis was employed to improve the logistic regression model. This iterative method refines the model by eliminating variables with less relevancy to the outcome, while maintaining meaningful independent variables. The variables that were iteratively removed from the model were (in this order): Inseminated sperm; Infertility duration; Period from trigger to IUI; Male age; Female age; Type of infertility. Therefore, the final model encompasses the Female smoking habits, Infertility diagnosis, Mature follicles, Endometrial thickness and Normal morphology sperm variables (**Table 17**).

Table 17: Binomial logistic regression model after backwards elimination. CI- confidence interval; OR- odds ratio; ref- reference category; SE- standard error. **p-value<0.05; *0.05 < p-value < 0.1.

Variable		β	SE	OR	95% CI for OR	p-value
Constant		-2.570	0.663	0.077	-	<0.001
Female smoking habits	Non-smoker	0.098	0.360	1.103	0.545-2.235	0.785
	Past smoker	0.272	0.285	1.313	0.750-2.297	0.340
	<10/day	0.764	0.319	2.147	1.149-4.014	**0.017****
	10-20/day	-0.740	1.039	0.477	0.062-3.656	0.476
	>20/day (ref)	1.00	-	1.00	-	-
Infertility diagnosis	Unexplained	2.064	0.835	7.881	1.534-40.497	**0.013****
	Mixed	0.002	0.366	1.002	0.488-2.054	0.996
	Partial tubal factor	1.372	0.733	3.942	0.938-16.572	0.061*
	Ovulatory factor	-0.318	0.487	0.728	0.280-1.891	0.514
	Endometriosis	-0.334	0.288	0.716	0.407-1.259	0.246
	Male factor (ref)	1.00	-	1.00	-	-
Mature follicles		0.247	0.150	1.280	0.954-1.716	0.099*
Endometrial thickness		0.086	0.049	1.090	0.990-1.200	0.081*
Normal morphology sperm		-0.019	0.011	0.981	0.960-1.002	0.073*

Significant coefficients (p-value<0.05) were obtained for the <10 cigarettes/day category (β=0.764; OR= 2.147; p-value= 0.017) of the Female smoking habits variable (reference category: >20 cigarettes /day), and the Unexplained category (β=2.064; OR= 7.881; p-value= 0.013) of the Infertility diagnosis variable (reference category: Male factor). The positive β values for both categories indicate that the log-odds of successful outcome are higher for those categories, compared to the reference category from the correspondent variables. Specifically, based on the OR values, it is possible to conclude that female patients belonging to the <10 cigarettes/day category have ~2 times higher odds of achieving clinical pregnancy than female patients who belong to the >20 cigarettes/day category, and cases diagnosed with unexplained infertility have ~7 times higher odds of achieving clinical pregnancy than male factor infertility cases.

Marginally significant coefficients (0.05 < p-value < 0.1) were obtained for the Mature follicles (β=0.247; OR= 1.280; p-value= 0.099), Endometrial thickness (β=0.086; OR= 1.090; p-value= 0.081) and Normal morphology sperm (β=-0.019; OR= 0.981; p-value= 0.073) variables. The positive β value and OR>1 for Mature follicles and Endometrial thickness denote a positive relationship between these variables and

clinical pregnancy, while the negative β value and OR<1 for Normal morphology sperm suggests a negative relationship with the successful outcome. Additionally, the partial tubal infertility category (β=1.372; OR= 3.942; p-value= 0.061) of the Infertility diagnosis variable also implied a marginally significant ~4 times increase in odds of achieving clinical pregnancy in cases diagnosed with partial tubal infertility, compared to male factor infertility cases.

The computed Hosmer-Lemeshow goodness-of-fit test yielded a chi-square value of 10.340, with 8 degrees of freedom and a corresponding p-value of 0.242. This non-significant result indicates that the logistic regression model has an adequate fit to the data. The calculated Nagelkerke pseudo-R^2 for the final model was 0.045, indicating that the independent variables explain ~4.5% of the variance in the log-odds of the outcome variable.

4. Discussion

Pregnancy is a highly complex process, that relies on many interplaying factors, all necessary for achieving the desired outcome. Studies that focus on finding the predictors for pregnancy in the context of IUI treatment often discover that the optimal scenario for successful outcome involves a set of contributing factors, both male and female, as well as specific cycle characteristics[452]. Li et al. report that an optimised IUI cycle should encompass ovarian stimulation to induce the growth of 1-2 follicles and a sperm sample with ≥4 million motile sperm[270]. Merviel et al. reports that female age <30 years old, infertility aetiology of cervical or anovulatory factor, ≥ 5 million motile sperm and recruitment of two follicles measuring ≥16 mm in diameter are the parameters that yield a better chance of pregnancy[453]. Dinelli et al. reports that optimal IUI cycles in women aged ≤38 years include infertility aetiology of ovulatory factor, normal ovarian reserve or secondary infertility, ≥1 million motile sperm and growth of two follicles[409]. These heterogenous findings reveal the complex nature of the study to optimise IUI pregnancy rates.

Even though female age and sperm count are examples of factors that are used as thresholds in ART laboratories, they were excluded from the binomial logistic regression model during the backward elimination procedure in this study. It is worth noting that when female age is reported as a significant factor for CP following IUI, the range of ages included in the correspondent studies is over 40 years and older[398,401,402,404,452]. However, when only women ≤40 years old are included, a significant relationship with CP is not obtained[416]. Additionally, 40 years old has been appointed as the cut-off age at which IUI is no longer recommended over IVF, due to the decreasing success rates[60,273]. Therefore, the absence of a significant relationship between female age and CP following IUI in the present dataset may be explained by the applied inclusion factors, that only range women from 19 to 40 years old. It is then hypothesised that if the data from cases with women aged 40-42 years old was included, this parameter would then be uncovered as significant.

Despite being one of the factors that are considered more relevant to CP after IUI cycles, inseminated sperm count is not consistently reported to be significant[270,398,405]. Thus, although it remains an expected significant parameter, it is not completely unforeseen that it is not in this dataset. It should be emphasized that non-significant results have been reported in studies that employ the post-wash total motile sperm count variable as a numerical variable[398,411], while studies with significant

outcomes often employ it as a categorical variable with several categories[270,404,405,408] or with two categories, above and below a certain threshold (such as 5 million)[402,407]. Therefore, a next step in this study would be to employ the Inseminated sperm count variable as categorical, which would also be valuable for confirming or redefining the present threshold that is used in the UMR-HSM laboratory for IUI execution or cancellation (1 million sperm cells).

In the Infertility diagnosis variable, the unexplained infertility aetiology was found to have significantly higher odds of achieving CP compared to male factor infertility in the present dataset. As stated earlier, previous reports do not shed a clear light on which of these two aetiologies is associated with higher odds of CP[405,411,435]. IUI is advised as a first-line treatment for couples diagnosed with unexplained infertility[454,455], but that same applicability for mild male factor cases is debated[436,437]. Given that the present study focuses on first IUI attempts, the obtained result corroborates the approach of using IUI as a first-line treatment for unexplained infertility. The partial tubal factor category exhibited a marginally significant increase in odds of achieving CP compared to male factor infertility. Considering that similar IUI outcomes between unilateral tubal occlusion and unexplained infertility patients have been reported[456,457], it would be expected that the obtained increase would be significant when the reference category is male factor infertility, taking into account what has been discussed. However, it is worth noting that this resemblance with unexplained infertility outcomes has been differentiated for unilateral proximal tubal occlusion and unilateral distal tubal occlusion, since the latter is associated with a lower success rate[456,458]. Therefore, it would be important to add that information to the dataset in further studies, to confirm if the marginal significance may derive from the inclusion of both occlusions together. Lastly, it would be expected for the ovulatory factor category to yield a significant result, since ovarian stimulation can bypass the anovulation/ovulation dysfunction aetiologies[125], and IUI usually achieves better outcomes in these cases[409,432,433]. In the case of the present study, the absence of a significant relationship may be a reflection of the low number of cases with ovulatory factor in the dataset (9/1077).

The Female smoking habits variable was kept in the final model, and yielded a statistically significant result for the <10 cigarettes/day category, compared to the >20 cigarettes/day reference category. This finding is in agreement with previous reports (1-14 cigarettes/day with ≥15 cigarettes/day as the reference)[431] and may be useful for patient counselling. While it is established that smoking habits have detrimental effects

on both female and male fertility[91,93,95], a definite relationship with reproductive outcomes remains elusive. Therefore, it is not surprising that the remaining categories (non-smoker, past smoker and 10-20 cigarettes/day) did not achieve the significance level.

Sperm morphology is widely studied as a factor influencing IUI outcomes, but current studies usually apply the Kruger's strict criteria described in the World Health Organisation (WHO) manual for examination of human semen[112,459]. Since the criteria for sperm morphology assessment used in the UMR-HSM laboratory are the previous, WHO 1992 reference criteria[445], studies reporting results for this parameter should use the same criteria for fair comparison. This approach led to a study that reported no differences in PR in cycles with ≥30% or <30% of normal forms, as well as a difference in PR according to the number of inseminated sperm only in cases with morphology below the threshold (<30%)[460]. This supports the notion that morphology should not be taken as an independent predictor of success after IUI, and rather should be considered together with other parameters[412,414]. In the case of the present study, a marginally significant negative association was found between the percentage of normal forms and CP after IUI, with a one-unit increase in the morphology value being associated with 0.981 times decrease in the odds of CP occurring, which represents merely a residual reduction. The expected result would be the opposite, with an increase in the odds of CP with an increase of normal forms, due to the association between sperm morphology and sperm quality and function[112,140], and previous reports of positive association[403,413]. However, a previous study using the strict criteria also uncovered an increase in the odds of CP after a first IUI cycle in cases with lower morphology (≤4% compared to >4%) and number of inseminated motile spermatozoa of 5-10 million[461], suggesting that cases of moderate male factor benefit from IUI treatment. To explore this correlation in the present dataset, a more in-depth study on the Normal morphology sperm and Inseminated sperm variables would have to be conducted.

It is relevant to keep in mind that sperm morphology evaluation is of highly subjective nature[462], which may yield results that lead to a loss of predictive power of this parameter[463]. Moreover, the switch to Kruger's strict morphologic criteria in WHO manuals was based on the predictive power of this scoring system for sperm quality and fertilising potential[112,142,149,459], which point to the employment of the Kruger's strict criteria for better prognostic value[462].

The Mature follicles variable also exhibited a marginally significant association with CP after IUI in the current study, with an increase of one follicle being associated with 1.280 times increase in the odds of CP occurring. This result is in accordance with the literature[268,270] and showcases the established conception of greater PR in IUI cycles with a higher number of developing follicles after ovarian stimulation, although the recognition that safety must be prioritized underscores the goal of limiting the number of accepted maturing follicles to a maximum of two. It would be expected for this variable to have a significant impact on CP odds, but the marginally significant increase found in the present study may be a reflection of the skewed distribution present in the variable (1 follicle: 662 cases; 2 follicles: 357 cases; 3 follicles: 49 cases; 4 follicles: 5 cases), that can influence coefficient and significance estimation[464].

In the current study, another variable that displayed a marginally significant link with CP following IUI was Endometrial thickness, with a one-unit increase in thickness being associated with 1.090 times increase in the odds of CP occurring, which represents merely a residual increase. This marginally significant residual increase of CP odds may be a reflection of the non-significant difference between endometrial thickness between the pregnant and non-pregnant groups in the present study (**Table A1**) (Non-pregnant: 9.76 ± 1.98; Pregnant: 10.03 ± 1.70; p-value: 0.094). The substantial variability observed in reported results precludes the consideration of endometrial thickness as a predictor of IUI success by several authors[465–467]. Therefore, it was not expected that this parameter would yield a relevant association in the multivariate analysis.

These marginally significant ($0.05 < p\text{-value} < 0.1$) results that were obtained for some variables/categories should not be taken as predictors for IUI success, as they uncover possible associations that should be further investigated.

The obtained Nagelkerke pseudo-R^2 for the final model entails that the model explains a relatively small proportion of the variation in the log-odds of the outcome (~4.5%), and may be lacking in variables that play a substantial role in determining clinical PR following IUI, thereby explaining the remaining variation[442,449]. Additional potential patient-specific contributors can be other lifestyle factors, environmental factors and genetic factors. Cycle characteristics such as the ovarian stimulation drug, catheter types and post-insemination resting[400] could also be advantageous.

This study can be considered as an initial approach into the study of the most relevant contributors to IUI success within the UMR-HSM, marking the starting point of a more comprehensive and targeted investigation aimed at generating specific insights.

The end objective is to enhance the individualized optimization of each IUI cycle, with the prospect of improving pregnancy rates for this Assisted Reproductive Technology treatment. Subsequent studies can further inspect how the influence of the analysed parameters may vary when the dataset is segmented into groups based on a single parameter, such as Inseminated sperm count, Female age and/or Infertility diagnosis.

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Attachments

A. Statistical analysis

Table A1: T-test and chi-square test results for the initial set of variables, grouped by clinical pregnancy outcome. Variables with p-value >0.9 were excluded, due to weaker relevancy for clinical pregnancy. SD- standard deviation. *p-value<0.05; p-value>0.9 is in **bold**; †≤1% missing values; ‡≈25% missing values; ◊homogeneity of variances not assumed.

Variable		N (%) or Mean ± SD		t or χ^2	p-value
		Non-pregnant (N= 942)	Pregnant (N= 135)		
Female age (years)		34.41 ± 3.87	34.23 ± 3.80	0.514	0.608
Female BMI (kg/m2)		24.19 ± 4.47	24.15 ± 4.50	0.107	**0.915**
Female smoking habits†				5.947	0.203
	Non-smoker	679 (72.7%)	89 (66.4%)		
	Past smoker	74 (7.9%)	10 (7.5%)		
	<10/day	107 (11.5%)	18 (13.4%)		
	10-20/day	56 (6.0%)	15 (11.2%)		
	>20/day	18 (1.9%)	2 (1.5%)		
Male age (years)		35.82 ± 5.08	35.97 ± 5.515	-0.311	0.756
Male BMI‡ (kg/m2)		26.4 ± 3.59	26.02 ± 3.43	0.948	0.344
Male smoking habits‡				1.254	0.869
	Non-smoker	403 (56.9%)	55 (60.4%)		
	Past smoker	85 (12.0%)	9 (9.9%)		
	<10/day	82 (11.6%)	11 (12.1%)		
	10-20/day	99 (14.0%)	13 (14.3%)		
	>20/day	39 (5.5%)	3 (3.3%)		
Type of infertility†◊		1.30 ± 0.46	1.35 ± 0.48	-1.071	0.286
Infertility diagnosis†				12.224	0.032*
	Unexplained	645 (68.8%)	93 (68.9%)		
	Male factor	171 (18.2%)	21 (15.6%)		
	Partial tubal factor	65 (6.9%)	10 (7.4%)		
	Endometriosis	49 (5.2%)	5 (3.7%)		
	Ovulatory factor	6 (0.6%)	3 (2.2%)		
	Mixed	3 (0.3%)	3 (2.2%)		
Stimulation total dosage† (IU)		530.51 ±	531.58 ±	-0.044	**0.965**

	257.03	289.81		
Mature follicles†	1.43 ± 0.60	1.52 ± 0.65	-1.728	0.084
Endometrial thickness†◊ (mm)	9.76 ± 1.98	10.03 ± 1.70	-1.681	0.094
Period from trigger to IUI† (hours)	35.08 ± 3.42	34.87 ± 3.45	0.646	0.519
Inseminated sperm count (million)	10.72 ± 10.18	10.25 ± 9.78	0.504	0.614
Normal morphology sperm† (%)	35.25 ± 9.76	33.63 ± 10.20	1.782	0.075

T-test for numerical variables, and chi-square test for categorical variables. According to the Shapiro-Wilk test, none of the numerical variables present a normal distribution, but due to the number of cases within the analysis (>30 in each group), it is possible to apply the parametric t-test while still ensuring the robustness of the test (according to the Central Limit Theorem[468]). For the χ^2 test, <20% of the cells in the cross-tabulation had a count of less than 5 for all variables.

Table A2: Spearman coefficients for correlations between pairs of numerical independent variables. Coefficients of >0.7 were considered to indicate a strong relationship between variables[469].

Spearman coefficient	Female age	Female BMI	Male age	Male BMI	Infertility duration	Type of infertility	Stimulation total dosage	Mature follicles	Endometrial thickness	Period from trigger to IUI	Inseminated sperm	Normal morphology sperm
Female age	1	-0.007	0.510	0.002	-0.103	0.205	0.093	0.023	0.019	0.103	0.016	0.027
Female BMI		1	0.0240	0.265	0.150	0.072	0.203	-0.066	0.097	0.034	-0.052	-0.024
Male age			1	0.065	-0.005	0.130	0.022	0.019	0.020	0.118	-0.031	0.012
Male BMI				1	0.049	0.017	0.074	0.029	0.032	0.032	-0.010	-0.005
Infertility duration					1	0.059	0.060	-0.036	0.086	-0.056	-0.013	-0.065
Type of infertility						1	0.021	0.004	0.064	0.005	0.066	0.065
Stimulation total dosage							1	-0.031	-0.012	0.038	-0.046	-0.036
Mature follicles								1	0.133	-0.052	-0.054	-0.058
Endometrial thickness									1	-0.017	0.000	0.007
Period from trigger to IUI										1	-0.062	0.046
Inseminated sperm count											1	0.508
Normal morphology sperm												1

The non-parametric Spearman coefficient was chosen due to absence of normal distribution[469]. All of the pairs of variables demonstrated a Spearman coefficient lower than 0.7, indicating that they should be included in the Binomial logistic regression, and will contribute in a distinct way to the model.