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Rauni Klami

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ABSTRACT

Azoospermia is defined by a complete absence of sperm in repeated semen analyses. In non-obstructive azoospermia (NOA) sperm production in the testicles is severely impaired or completely absent. Sperm can be recovered in approximately half of the men with NOA by microdissection testicular sperm extraction (MD-TESE), and many of these men can father biological children with intracytoplasmic sperm injection (ICSI) (Corona et al. 2019). Assisted reproduction techniques (ART) using these sperm seem to be as effective as other treatments performed for moderate or severe male infertility (Ravizzini et al. 2008). Predicting sperm recovery and the ICSI treatment results for an individual with NOA remains difficult, and refraining from offering the treatment is rarely relevant (Enatsu et al. 2016).

In Finland, MD-TESE operations were started in 2008 at Turku University Hospital (Tyks). From the first 100 operations, information was collected on the patients' diagnoses, previous procedures to find sperm, and the results and adverse effects of the operations. Sperm were found in 42 men. ICSI treatments with MD-TESE sperm and other ICSI treatments for moderate or severe male infertility in Tyks were compared, and the treatment results were similar in terms of fertilization, clinical pregnancy rate, live birth rate, gestational age at delivery and birth weight of the children born after ICSI.

In the magnetic resonance imaging (MRI) of the testicles, the apparent diffusion coefficient (ADC) value, which reflects the metabolism of the tissue, can be measured (Tsili et al. 2018). Twenty-one men presenting for MD-TESE and ten fertile control men MRI of the pelvis, and testicular ADC values were compared in different diagnostic groups and in relation to sperm retrieval. There were no significant differences in ADC values in relation to sperm retrieval success in MD-TESE or between different diagnosis groups, but ADC values in healthy controls were significantly lower than in men with NOA.

This study found that MD-TESE is an effective way to recover sperm and achieve biological parenthood for men with NOA, but for more than half of the men, no sperm production exists in the testis to enable successful sperm recovery. New methods of predicting sperm recovery are needed to avoid unnecessary operations.

KEYWORDS: Azoospermia, Male Infertility, Non-obstructive azoospermia, MD-TESE

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TIIVISTELMÄ

Azoospermia tarkoittaa siittiöiden täydellistä puuttumista toistetuissa siemennesteanalyyseissa. Non-obstruktiivisessa azoospermiassa (NOA) kivesten siittiötuotanto on erittäin vähäistä tai puuttuu kokonaan. Hiukan alle puolelta miehistä on NOA-tilanteessakin löydettävissä siittiöitä kiveksen mikrodissektioleikkauksella (MD-TESE), ja moni heistä voi saada biologisia jälkeläisiä mikroinjektiohoidolla (ICSI) (Corona et al. 2019). Hedelmöityshoidot vaikuttavat yhtä tehokkailta kuin muutkin miehen kohtalaisen tai vaikean lapsettomuuden vuoksi tehdyt hoidot (Ravizzini et al. 2008). Siittiöiden löytymisen ja hedelmöityshoidon tulosten ennustaminen yksittäisen miehen NOA-tilanteessa on vaikeaa, ja hoidosta pidättäytyminen on siksi vain harvoin perusteltua (Enatsu et al. 2016).

Suomessa MD-TESE-leikkaukset aloitetiin vuonna 2008 Turun yliopistollisessa keskussairaalassa (Tyks). Sadasta ensimmäisestä leikkauksesta kerättiin tiedot potilaiden diagnooseista, edeltävistä toimenpiteistä siittiöiden löytämiseksi sekä leikkausten tuloksista ja haittavaikutuksista. Verrattaessa MD-TESE-siittiöillä Tyksissä tehtyjä ICSI-hoitoja muihin miehen kohtalaisen tai vaikean lapsettomuuden vuoksi tehtyihin ICSI-hoitoihin, hoitotulokset olivat samanlaiset.

Kivesten magneettitutkimuksessa (MRI) voidaan mitata kudoksen aineenvaihduntaa kuvastava apparent diffusion coefficient -arvo (ADC) (Tsili et al. 2017). 21 MD-TESE-leikkaukseen saapuvalle miehelle ja kymmenelle hedelmälliselle verrokkimiehelle tehtiin lantion magneettikuvaus (MRI), ja kivesten ADC-arvoja verrattiin eri diagnoosiryhmissä ja suhteessa siittiöiden löytymiseen. ADC-arvoissa ei ollut eroja suhteessa siittiöiden löytymiseen MD-TESE-leikkauksessa eikä diagnoosiryhmien välillä, mutta verrokkien ADC-arvot olivat matalampia kuin miesten, joilla oli NOA.

Tutkimus osoitti, että MD-TESE on tehokas tapa löytää siittiöitä ja saavuttaa biologinen vanhemmuus NOA-tilanteessa, mutta yli puolelta leikkaukseen tulevista miehistä ei löydy kiveksestä siittiötuotantoa. Uusia menetelmiä siittiöiden löytymisen ennustamiseen tarvitaan, jotta vältyttäisiin turhilta leikkauksilta.

AVAINSANAT: atsoospermia, lapsettomuus, non-obstruktiivinen atsoospermia, MD-TESE

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Abbreviations

AAS	Anabolic androgenic steroid
ADC	Apparent diffusion coefficient
AI	Aromatase inhibitor
AZF	Azoospermia factor
AMH	Anti-Mullerian hormone
BMI	Body mass index
CC	Clomiphene citrate
cLBR	Cumulative live birth rate
CPR	Clinical pregnancy rate
DWI	Diffusion-weighted imaging
FET	Frozen embryo transfer
FSH	Follicle stimulating hormone
hCG	Human chorionic gonadotropin
hMG	Human menopausal gonadotropin
HH	Hypogonadotropic hypogonadism
ICSI	Intracytoplasmic sperm injection
KS	Klinefelter syndrome
LBR	Live birth rate
LH	Luteinizing hormone
MA	Maturation arrest
MD-TESE	Microdissection testicular sperm extraction
MR(I)	Magnetic resonance (imaging)
MTR	Magnetic transfer ratio
NOA	Non-obstructive azoospermia
OA	Obstructive azoospermia
OAT	Oligoasthenoteratozoospermia
PVP	Polyvinylpyrrolidone
PR	Pregnancy rate
SA	Spermatogenic arrest
SERM	Selective estrogen receptor modulator
SRR	Sperm recovery rate

TESA	Testicular sperm aspiration
TESE	Testicular sperm extraction
TSH	Thyroid-stimulating hormone
TTP	Time to pregnancy
TYKS	Turun yliopistollinen keskussairaala, Turku University Hospital
WHO	The World Health Organization

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Klami R, Mankonen H, Perheentupa A: Microdissection testicular sperm extraction in Finland - results of the first 100 patients. *Acta Obstet Gynecol Scand.* 2018 Jan;97(1):53–58.

II Klami R, Tomás C, Mankonen H, Perheentupa A: Male ICSI outcome after microdissection testicular sperm extraction, testicular sperm aspiration and ejaculated sperm. *Reproductive Biology*. 2024 Mar; 24(1).

III Klami R, Pärssinen H, Sainio T, Blanco Sequeiros R, Perheentupa A: Can testicular apparent diffusion coefficient predict the presence of spermatogenesis in men undergoing microdissection testicular sperm extraction for non-obstructive azoospermia? *Manuscript*

1 Introduction

Infertility is a very common condition, affecting approximately 17.5% of the couples trying to conceive (Cox et al. 2022). Infertility is defined as the failure to achieve a pregnancy after more than 12 months of unprotected regular intercourse. It is estimated that globally more than 186 million individuals live with infertility, affecting their mental health, quality of life and social status (Inhorn and Patrizio 2015). Infertility can be caused by female and/or male factors, or it can be unexplained. The male factor is thought to be present in approximately half of the infertility cases (Cox et al. 2022).

The semen sample is the first line test for couples suffering from infertility (Sharlip et al. 2002). The WHO laboratory manual for the examination and processing of human semen gives detailed instructions on sample collection and processing (Björndahl, Kirkman Brown et al. 2022). A deviant result should always be repeated, as the semen quality varies greatly (Colpi et al. 2018). The complete lack of spermatozoa or azoospermia in repeated sperm analyses is present in approximately 1% of all adult males and 10–15% of infertile males (Willott 1982). In obstructive azoospermia (OA) the spermatogenesis is normal, but no spermatozoa are present in semen due to an obstruction or congenital abnormality of the genital tract. The most severe form of male infertility is non-obstructive azoospermia (NOA), where the spermatogenesis is severely impaired.

Intracytoplasmic sperm injection (ICSI, (Palermo et al. 1992)) has enabled biological fatherhood for infertile males since the 1990s. It is a technique where a single sperm is micro-injected into an oocyte, making fertilization possible with very small numbers of spermatozoa. In OA, an epididymal or testicular sperm extraction (TESE) or aspiration (TESA) needle biopsy combined with ICSI is a successful treatment option (Silber et al. 1995; Devroey et al. 1995), but in NOA, these biopsies are very often unsuccessful. The spermatogenesis is in most NOA cases present in small areas of the testes, if any. Microdissection testicular sperm extraction (MD-TESE) is a technique that enables these areas to be visualized with an operating microscope, enabling sperm collection in approximately half of NOA cases (Schlegel 1999).

In Finland, Turku University Hospital Fertility Unit was the first hospital to introduce MD-TESE as a treatment option for NOA in 2008. Before this, donor sperm treatment was the only treatment option, and the availability of donor sperm was poor in publicly funded clinics due to the 2007 Finnish Law on Assisted Reproduction, banning anonymous gamete donation. Most Finnish clinics started referring their NOA patients to Turku within a few years after the MD-TESE operations began.

MD-TESE and ICSI treatment following sperm recovery in MD-TESE are expensive treatments requiring trained and experienced staff as well as equipment to be carried out successfully. Predicting the sperm recovery rate (SRR) in MD-TESE and the chance of fathering a biological child remains very difficult (Caroppo and Colpi 2021b). Accurate data should be made available on SRR, live birth rate (LBR), costs and safety of the treatment not only for the use in pre-surgery individual patient counselling but also for the use and evaluation of the public health system.

2 Review of the Literature

2.1 Male Fertility

2.1.1 Overview of the male reproduction

Male fertility is a complex, multifactorial entity. In the Finnish population, the total fertility rate is declining, which currently means that 27% of Finnish men will never father a child (Stat.fi ISSN=1798-2391. Helsinki: Tilastokeskus.). Semen analysis parameters also show a declining pattern over the past decades, leading to concern over male fertility (Rodprasert et al. 2019). Several genetic and environmental factors causing male infertility and subfertility have been found (Skakkebaek et al. 2016).

Gender differentiation into a male is a complex and error-prone process that requires the presence of the Y chromosomal SRY (sex-determining region of Y chromosome) gene and hormonally appropriate circumstances. The anti-Müllerian hormone (AMH) produced by Sertoli cells, and the testosterone produced by Leydig cells control the development of the male urinary and genital organs. The fetal hypothalamic-pituitary axis begins to regulate the developing testes that are initially controlled by human chorionic gonadotropin (hCG). Y-chromosome microdeletions and Klinefelter's syndrome (47, XXY) are the most well-known genetic causes of male infertility (Okutman et al. 2018). Other causes include genetic abnormalities associated with sex differentiation, steroidogenesis, and steroid hormone metabolism. Moreover, a pregnant mother’s lifestyle, health, and living environment during pregnancy may be even more important for the future fertility of the child than his lifestyle (Juul et al. 2014).

In puberty, the testis starts to produce testosterone and spermatozoa. Spermatogenesis is a 64-day process activated in seminiferous tubules, where the diploid germ cells or spermatogenic stem cells will be developed into haploid mature spermatozoa by going through a mitotic division and two meiotic divisions, and morphological transformation during haploid differentiation (Heller and Clermont 1963). As the number of Sertoli cells, which are important for germ cell metabolism and communication, does not increase during a man's fertile age, the number of Sertoli cells is a limiting factor for the sperm cell number (Clermont 1972).

Spermatogenesis is possible only at temperatures below body temperature, making the descent of the testes essential for fertility. Spermatogenesis also requires endocrine regulation, especially the effect of follicle-stimulating hormone (FSH) and a high intratesticular testosterone level (McLachlan et al. 1996).

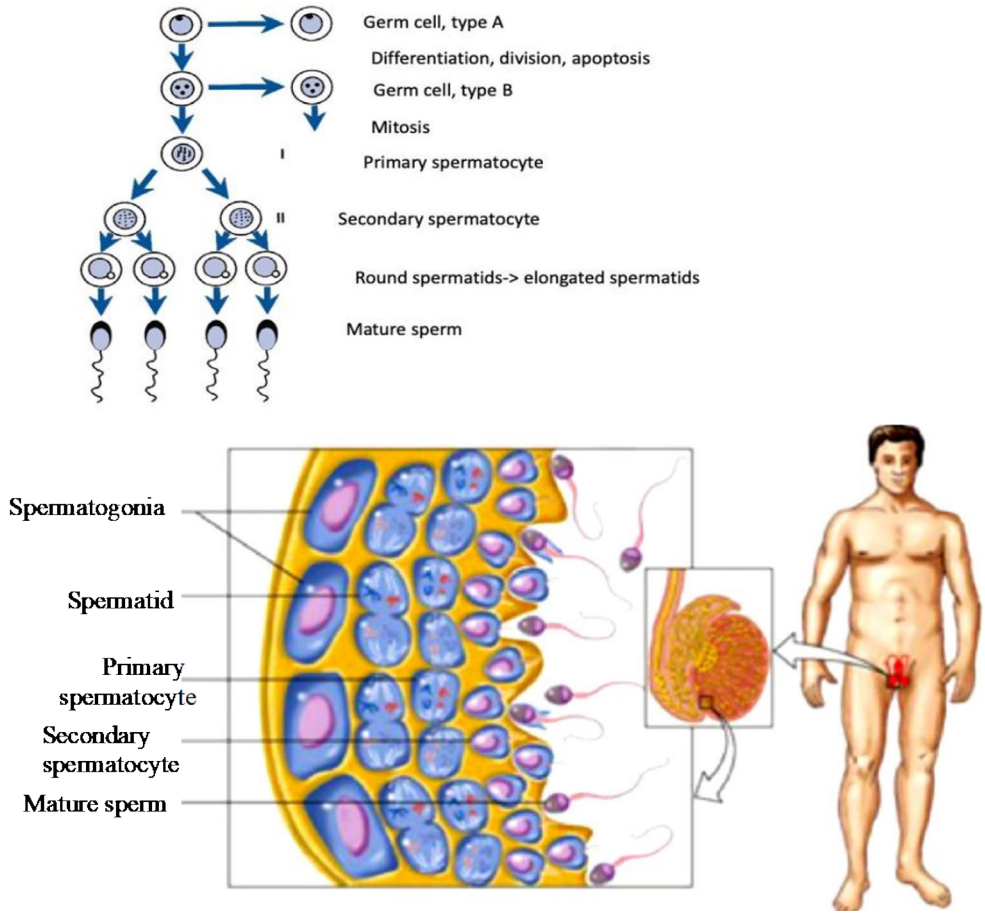

Figure 1. Spermatogenesis. I = first meiotic division, II = second meiotic division. Klami et al, Finnish medical journal 2021. Reprinted with permission from the copyright holder.

2.1.2 Assessing male fertility

Semen analysis is the best known and the first test of male fertility, whose ability to predict the onset of pregnancy is however quite poor. Natural conception is often reached despite a very abnormal semen analysis, while a normal result does not guarantee male fertility. An abnormality in a single parameter has been found to delay the onset of pregnancy only slightly, while an abnormality in several

parameters significantly increases the risk of infertility. When interpreting an abnormal sperm analysis, repeating the sperm analysis is the first procedure, as there is considerable variation in the semen parameters of an individual male (Schwartz et al. 1979).

Table 1. Semen analysis diagnoses explained. Adapted from Duodecim 2015 (Klami and Perheentupa 2015).

Normospermia	Normal semen
Oligozoospermia	Sperm concentration below normal range
Azoospermia	No sperm in semen
Asthenozoospermia	Motility below normal range
Teratozoospermia	Sperm morphology abnormal
Cryptozoospermia	Viability abnormal
Leukospermia	White blood cells in semen
Hematospermia	Red blood cells in semen
Aspermia/ hypospermia	No semen/ Low semen volume
Oligoasthenoteratozoospermia	Abnormal sperm count, motility and morphology

There are several commercial tests measuring sperm DNA fragmentation and oxidative stress. Sperm stored in the epididymis are susceptible to DNA fragmentation, the main cause of which is oxidative stress (Smits et al. 2019). Sperm DNA fragmentation increases with male ageing and is more prevalent in overweight and smoking men. An increase in DNA damage is also seen in genital infections. DNA damage has been found to reduce the chance of spontaneous pregnancy and childbirth, impair the outcome of infertility treatment, and increase the risk of miscarriage. There is preliminary evidence for the benefit of the use of antioxidants in improving sperm DNA structure and fertility (Bisht et al. 2017). The effectiveness of lifestyle changes is less well documented. Short abstinence is associated with less sperm DNA fragmentation, and the extent of DNA fragmentation is lower in testicular sperm than in ejaculated sperm. A healthy oocyte of a young female has the ability to partially repair sperm DNA fragmentation, and thus the advanced female age increases the impact of DNA fragmentation on a couple's fertility (Meseguer et al. 2011). Sperm DNA fragmentation testing may be warranted in specific groups, but not as a routine test of male fertility. Comparing the studies is difficult since there are several different commercial DNA fragmentation test methods (Esteves et al. 2015).

2.1.3 Factors affecting male fertility

Male fertility, in contrast to female fertility, has the potential to last throughout the lifetime. The ageing of the germ cells and the testicular somatic cells, as well as the hormonal changes, will however lead to a decrease in sperm production starting at 45 years of age. DNA repair mechanisms will deteriorate and de novo -mutations occur more frequently. There is convincing evidence showing an increase in miscarriage rate and incidence of certain diseases in the offspring of older fathers (Paul and Robaire 2013; du Fossé et al. 2020).

Many lifestyle factors have been shown to be associated with male fertility parameters, but the effects of lifestyle changes in interventional studies have failed to show an improvement in time-to-pregnancy of the female partner (Salas-Huetos, Bulló, and Salas-Salvadó 2017). Diet seems to have implications for semen quality, sperm DNA damage, and time to pregnancy (TTP). A healthy diet high in omega-3 fatty acids and some antioxidants (vitamins E and C, β-carotene, selenium, zinc and lycopene) and low in trans fats and saturated fats is linked to good semen parameters and short TTP (Salas-Huetos, Bulló, and Salas-Salvadó 2017), but data on the effects of diet changes is lacking. Obesity affects the function of the hypothalamic-pituitary axis and thus fertility. Chronic inflammation and sperm DNA fragmentation have been suspected to explain the poorer success rate in assisted reproductive technology (ART) in couples with paternal obesity (T. Glenn, Harris, and Lindheim 2019). Tobacco smoking is associated with impaired sperm production, but the mechanisms are somewhat unclear, as is the time required for the harmful effects to be reversed after smoking cessation (Sansone et al. 2018). Smoking is also associated with a higher DNA fragmentation index. Continuous and heavy alcohol use is linked to a decrease in serum testosterone level and an increase in serum estradiol and gonadotropin levels. Excessive alcohol consumption impairs semen parameters and increases sperm DNA fragmentation index (Muthusami and Chinnaswamy 2005).

The noted decline in semen quality since industrialization has raised concern about the impact of environmental factors on male fertility. Testicular dysgenesis syndrome is a term that refers to a group of urinary and genital malformations, such as cryptorchidism and testicular germ cell tumours, as well as impaired sperm production (Skakkebaek, Rajpert-De Meyts, and Main 2001). Environmental androgenic and estrogenic compounds appear to be particularly detrimental during fetal sex determination (Eisenberg et al. 2015; Skakkebaek et al. 2016). Numerous chemicals harmful to the fertility of experimental animals have been identified, but for humans, a causal link has only been shown for a few substances (Eskenazi et al. 2018). Known endocrine disrupting chemicals are common substances used in the manufacture of plastics, paints, surface treatments, insect control and firefighting (Giudice 2021).

Medication and general health affect fertility in several ways. On the other hand, abnormal semen quality is an independent risk factor for many diseases (Eisenberg et al. 2015). Metabolic syndrome, diabetes, hyperlipidemia, and cardiovascular mortality are more frequent in men with infertility. Poor semen quality is associated with an increase in overall mortality, the risk of hypertension, heart disease and stroke. Adequate medical treatment of underlying diseases has been shown to improve fertility (Shiraishi and Matsuyama 2018). Acute severe febrile infections, trauma, generalized diseases, and cancers may impair semen quality, most often by inhibiting gonadotropin secretion of the pituitary gland.

Testosterone treatment will, through direct downregulation of the pituitary gland, cease sperm production in most men, but often reversibly. Anabolic androgen use and abuse have the same effect (Drobnis and Nangia 2017b). 5α-reductase inhibitors for the treatment of benign prostatic hyperplasia and androgenic alopecia may impair semen quality, erection, ejaculation, and libido (Drobnis and Nangia 2017a). Many psychiatric drugs, most commonly antipsychotics, may increase serum prolactin level, leading to a decrease in testosterone production. Cytotoxic medication and radiation have an adverse effect on spermatogenesis. Fertility may take as long as 24 months to recover and highly depends on the dosage of radiation and the type and dosage of medication received. The potential effects of radiation and cytotoxic medication on the health and the reproductive capacity of the next generations are not completely understood (Paoli et al. 2016).

Varicocele is present in approximately 15% of males and 35–75% infertile males. Varicocele is associated with an increased risk of sperm abnormality, and surgical treatment has been shown to improve sperm parameters and, although less convincingly, pregnancy outcomes (A. Mehta and Goldstein 2013). The harmful mechanism of varicocele on testicular function is not fully understood, but circulation and abnormal testicular temperature are the most speculated factors. Varicocelectomy has been shown to reduce the incidence of sperm DNA damage. The presence of varicocele may be associated with elevated gonadotropin levels and, in some studies, lower testosterone levels, but the benefit of surgery for hormonal function is disputed (Damsgaard et al. 2016).

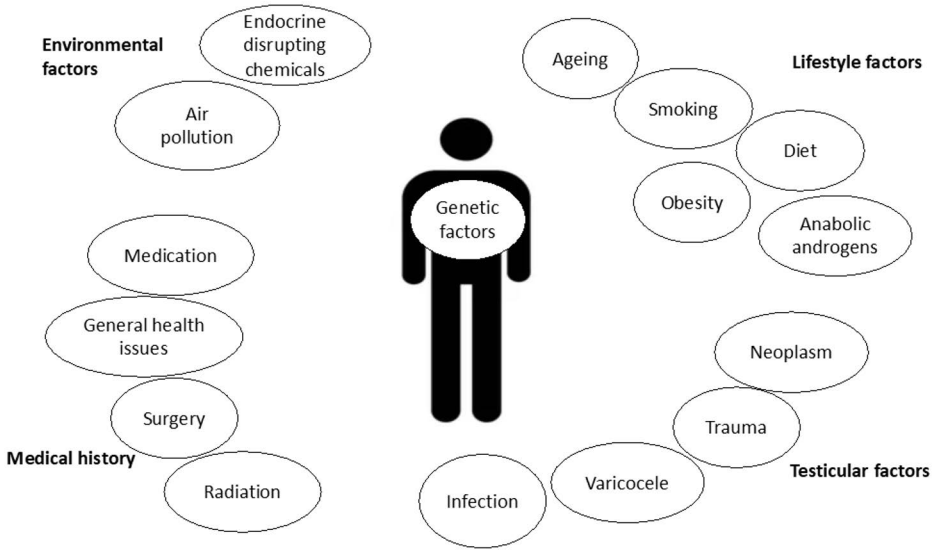

Figure 2. Factors that affect male fertility, as in paragraph 2.1.3.

2.2 Male infertility

2.2.1 Evaluating male subfertility

Male infertility is a contributing factor in up to 50% of infertile couples and considered the sole factor in 25 to 40% (Thoma et al. 2013; Thonneau et al. 1991). After a comprehensive work-up to examine the aetiology, 60 to 75% of male infertility will remain unexplained (Thonneau et al. 1991). In a study of 1767 infertile males attending a fertility clinic, 60% were unexplained, genetic causes explained 7,8%, congenital causes 10,7%, testicular 6,5%, seminal tract 6,0%, oncological causes 3,4%, secondary hypogonadism 1,3% and sexual dysfunction 4,4% (Punab et al. 2017).

Semen sample is the first line test for couples suffering from infertility. The WHO laboratory manual for the examination and processing of human semen gives detailed instructions on sample collection and processing (Björndahl, Kirkman Brown et al. 2022). An abnormal result should always be repeated, as the semen quality varies greatly. For the two most recent versions of the manual, data was assessed from fertile men who have achieved a natural pregnancy with a known TTP ≤ 12 months and a sexual abstinence period of 2–7 days. 5th centile of a sperm parameter was set as the lower limit of normal result. There is a considerable degree of overlap in the sperm concentrations of fertile and infertile males, underlining the fact that sperm analysis is a useful tool in male fertility studies, but a poor predictor of spontaneous pregnancy. The current WHO criteria are shown in Table 2.

Table 2. Normal values for the sperm parameters. WHO 2021(Björndahl, Kirkman Brown et al. 2022).

Normal values for sperm analysis, WHO 2021	
Semen volume (ml)	>1.4
Total sperm number (million per ejaculate)	>39
Total motility (%)	>42
Progressive motility (%)	>30
Normal forms (%)	>4

Male infertility examination should include detailed questionnaire on medical history, including pubertal timing, diet, lifestyle, sexual function, physical activity, occupational and other exposures, and family history. In the physical examination, pubic and body hair, weight, and height should be documented. A scrotal examination to measure testicular size, presence on of vas deferens, varicocele and to exclude a testicular mass should be carried out. Hormonal evaluation and genetic testing (microdeletions of the Y chromosome and karyotype analysis) are widely recommended, although most experts recommend genetic testing only in cases with severe oligozoospermia (Colpi et al. 2018). If all the pituitary hormone levels are low, magnetic resonance imaging of the pituitary is recommended. In cases with oligozoospermia or palpable testicular mass, a scrotal ultrasound examination is necessary.

2.2.2 Treatment of male subfertility

Medical treatment of male hypogonadism is needed when the serum gonadotropin levels, and serum testosterone level are low (hypogonadotropic hypogonadism, HH) or when the serum gonadotropin levels are not appropriately elevated compared to the low serum testosterone level. In HH, the treatment is usually started with human chorionic gonadotropin (hCG), and FSH may be added if the hormonal response is insufficient (Salenave et al. 2012). Aromatase inhibitors (AI, letrozole or anastrozole) and selective oestrogen receptor modulators (SERMs, clomiphene citrate (CC) or tamoxifen) are the peroral treatment options, only effective when the pituitary function is not severely impaired (Chua et al. 2013; Del Giudice et al. 2020; Yang, Li, and Li 2021). These medications diminish the oestradiol feedback to the pituitary and hypothalamus, causing increased gonadotrophin secretion. A novel treatment option showing promise in many recent trials, is FSH for men with idiopathic oligozoospermia but normal hormonal status. It has shown not only to improve semen parameters (Simoni et al. 2020; Santi, Granata, and Simoni 2015; Santi et al. 2020), but also DNA fragmentation index (Garolla et al. 2017).

Isotretinoin was also successful in significantly increasing sperm concentration in a small study of 19 men with oligoastenozoospermia (OAT) (Amory et al. 2017).

Correcting elevated serum prolactin (PRL) level with cabergoline has been shown to correct gonadal function (De Rosa et al. 1998). Leukocytes in sperm are an indicator of infection or inflammation in the urogenital tract and therefore often treated with antibiotics, but conclusive evidence on the benefit is lacking (Hamada et al. 2011). Retrograde ejaculation is a condition where the impaired bladder neck muscular function will leak the ejaculate into the bladder; in some studies with rather small sample size, sympathomimetics have been somewhat effective in correcting the condition (A. Mehta and Sigman 2015). Sexual dysfunction is a cause of male infertility often not shown in semen analysis. The infertility diagnosis per se may also have a detrimental effect on the psychosocial and sexual well-being (B.H. Luk and Loke 2015). Sexual therapy and psychosocial support are recommended. Medication for erectile dysfunction may be of assistance.

Testosterone and anabolic androgen steroid (AAS) use are common in adult male population (Drobnis and Nangia 2017b). Their deleterious effect on sperm production is dramatic, but often reversible (Turek et al. 1995). Discontinuing AAS is often difficult; tolerating the withdrawal symptoms such as insomnia, depression and erectile dysfunction may lead to lack of compliance and patient drop out (Drobnis and Nangia 2017b).

Intrauterine insemination (IUI) is considered the first line treatment option of mild to moderate male infertility (Bahadur and Homburg 2019) in view of its safety and cost-effectiveness (Bahadur et al. 2020). The success rate depends greatly on sperm parameters; the couples with an inseminated motile count (IMC) of less than 1 million should be advised against IUI (Ombelet et al. 2014). Long abstinence prior to IUI is not beneficial, and studies with pooled consecutive ejaculates have been successful. This finding may especially be of use in low resource settings (Gurunath, Gundlapalli, and Louis 2021). Female age and ovarian reserve should be assessed, as the IUI results depend on normal female fertility (Kamath et al. 2010).

Intracytoplasmic sperm injection (ICSI, (Palermo et al. 1992)) was originally introduced as a treatment for severe male factor infertility, where in vitro fertilization (IVF, (Steptoe and Edwards 1978) often failed. The use of ICSI is increasingly common in mild male factor as well. ICSI utilization has indeed spread beyond male factor infertility, although multiple large studies have shown that it does not increase the cumulative live birth rate in non-male factor infertility (T.L. Glenn, Kotlyar, and Seifer 2021). In mild male infertility (5 to 15 million spermatozoa per ml), there was no difference between IVF and ICSI in terms of fertilization rate (FR) or live birth rate (LBR, (Liu et al. 2020)). In the very frustrating event of failed fertilization, rescue ICSI has been studied with various results. In 2021 review and meta-analysis, only 5% implantation rate was achieved in rescue ICSI fresh embryo transfer, but

the transfer of cryopreserved rescue ICSI embryos resulted in 18% implantation rate (Paffoni et al. 2021).

2.2.3 Azoospermia

2.2.3.1 Definition of azoospermia

Azoospermia, complete lack of sperm in repeated semen analyses, is present in more than 10% of infertile couples and in 1% of general population of males (Willott 1982; R.H. Mehta et al. 2006). Azoospermia is divided into two categories; in obstructive azoospermia (OA) there is an obstruction in the urogenital duct system, but the sperm production is normal, while in non-obstructive azoospermia the sperm production is impaired. Both conditions can be congenital or acquired.

It is possible to make a clinical differential diagnosis between NOA and OA with very high sensitivity and specificity (Huang, Huang, and Lin 2017). Testicular size below average, elevated FSH, Klinefelter syndrome, Y chromosome microdeletions, history of cryptorchidism and previous treatment for malignancy are more often associated with classical NOA (Huang, Huang, and Lin 2017).

Regulatory causes of NOA are sometimes classified as their own subgroup of hypothalamic hypogonadism (HH), a condition with hypothalamic or pituitary malfunction, present in some NOA men. Low FSH and LH levels leading to low testosterone production cause an impairment of spermatogenesis, that can be successfully treated medically with hCG and, if necessary, FSH. The sperm analysis results should not be expected too early as spermatogenesis may take up to 6-24 months to fully recover (Ring, Lwin, and Köhler 2016).

2.2.3.2 Obstructive azoospermia

In obstructive azoospermia (OA), sperm production in the testis is normal, but the transportation of sperm to the semen is impaired. The most common of etiologies of OA include vasectomy, anatomical abnormality, infection, cystic fibrosis, or trauma (Miyaoka and Esteves 2013).

Before the wide use of ICSI in the 1990s, sperm donation was the only treatment option for severe male infertility. Shortly after ICSI was introduced, it became possible for the men with OA to achieve biological parenthood with ICSI with testicle or epididymal needle biopsy (Devroey et al. 1995; Silber et al. 1995).

Since the production of sperm is normal in OA, sperm can be recovered in the testis or the epididymis by testicular or epididymal aspiration in more than 90% of men (Carpi et al. 2011). There are numerous testicular biopsy techniques in use. In Finland, most fertility clinics use a punch biopsy instrument and perform testicular

extraction sperm aspiration in local anesthesia, using spermatic cord block. Sperm recovery rate (SRR) is good (90–100%) with all needle biopsies in OA (Miyaoka and Esteves 2013).

2.2.3.3 Non-obstructive azoospermia

NOA may be caused by an intrinsic testicular failure or inadequate gonadotropin production of the pituitary gland. The testicular causes include genetic factors, testicular neoplasm, undescended testes, previous testicular torsion, and environmental factors such as radiation, cytotoxic medication, and trauma. The etiologies and histopathological findings in NOA are discussed in detail in 2.4.3. Kallmann syndrome, pituitary surgery, medication (most commonly androgen use), and systemic disease are the most common causes of impaired regulation of testicular function. In a standard medical setting, a large proportion (20%) of NOA remains unexplained (Fedder et al. 2004).

The current knowledge of the monogenic causes of NOA is limited. The biggest challenge is the complex nature of spermatogenesis, leading to genetic heterogeneity. There are 38 established NOA genes that represent essential guardians of meiosis, transcriptional and endocrine regulators of reproduction. Approximately half of these NOA genes have also been found in ovarian insufficiency, amenorrhea, and certain female genital abnormalities, which implies there may be overlapping genetic mechanisms (Kasak and Laan 2021).

Needle biopsies described in paragraph 2.2.3.2 are inexpensive in terms of costs and scarce in complications, but their SRR is good only in OA (Bernie, Mata, et al. 2015). Using a larger needle in testicular biopsy may increase the SRR in NOA men (Carpi et al. 2011). Attempts have been made to improve TESA success in NOA by taking multiple samples (C.F. Jensen et al. 2016). In a recent randomized controlled trial, multiple TESA was shown to be significantly worse in sperm retrieval compared to MD-TESE (C.F.S. Jensen et al. 2022).

Conventional testicular sperm extraction (cTESE) is a technique where the testis is delivered from the scrotum, an incision is made on the tunica albuginea and several macroscopic biopsies are taken for examination, as described by Devroey in 1995 (Devroey et al. 1995) . The methods of testicular biopsy are pictured in Figure 3.

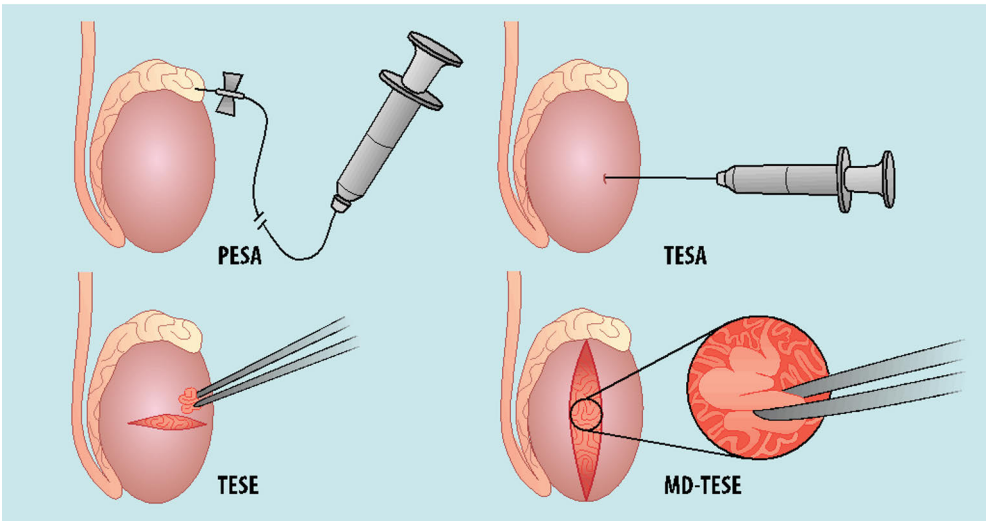

Figure 3. Testicular biopsy methods. PESA = percutaneous epididymal sperm aspiration, TESA = testicular sperm aspiration, TESE = testicular sperm extraction, MD-TESE = microdissection sperm extraction (Klami et al, Duodecim 2018, reprinted with permission from the copyright holder)

2.3 MD-TESE procedure

2.3.1 History of MD-TESE

When the MD-TESE procedure was first introduced in 1999 by Peter Schlegel (Schlegel 1999), the initial aim was to reduce the surgical complications associated with the conventional TESE (cTESE). This was accomplished by the ability to better avoid damage to the testicular vessels. However, the use of an operating microscope also assisted in identifying the heterogeneous structure of the seminiferous tubules, allowing the selective biopsies of the most eligible tubules in terms of sperm production. A noted improvement in sperm recovery rate (SRR) associated with this novel technique was soon observed (Schlegel 1999), and the method started to reach popularity globally a few years later.

2.3.2 Medical treatment prior to MD-TESE

At the first contact of an infertile couple, the role of the male partner is often overlooked. Patient evaluation should start with detailed interview of medical history, family history and lifestyle factors. The azoospermia should always be verified with repeating the sperm analysis. Physical examination and testicular ultrasound should be carried out since azoospermia and male infertility are risk factors for testicular cancer (Skakkebaek and Jørgensen 2005). Laboratory

evaluation for FSH, T, LH, inhibin-B, TSH, PRL and genetic testing for Y chromosome microdeletions and chromosomal abnormalities should be included in the examination (Rucker et al. 1998).

Intratesticular testosterone level as well as sufficient circulating FSH levels are essential for normal spermatogenesis. Up to 50% of the NOA men present with hypogonadism, showing low T level (Bobjer et al. 2012). A consensus exists to attempt to reach normal serum T level well in advance of MD-TESE (Reifsnyder et al. 2012).

Testosterone and anabolic androgen steroid use have become increasingly popular in healthy males, and the addictive nature of androgen effects makes discontinuing them potentially difficult (Mędraś, Brona, and Jóźków 2018). Exogenous testosterone administration suppresses the endogenous gonadotrophin levels and consequently depletes spermatogenesis in most men. Testosterone and other anabolic steroid use should therefore be discontinued prior to MD-TESE (McLachlan et al. 2002).

Hypogonadism treatment, if necessary, should be commenced 4 to 6 months prior to MD-TESE, due to the physiology of spermatogenesis. The aim is to improve the testicular testosterone production to create an appropriate hormonal milieu for spermatogenesis. Aromatase inhibitors (AI, letrozole or anastrozole) and selective oestrogen receptor modulators (SERMs, clomiphene citrate (CC) or tamoxifen) are the peroral treatment options. These medications diminish the oestradiol feedback to the pituitary and hypothalamus, causing increased gonadotrophin secretion. In a recent study (Alrabeeah et al. 2021), no benefit was shown from CC administration prior to MD-TESE. On the other hand, a study combining CC with hCG and human menopausal gonadotropin (hMG) did show improved SRR compared to the control group (Hussein et al. 2013).

The hormonal response to SERMs or AI is monitored by measuring circulating LH and T concentrations. In cases with lacking pituitary response or suboptimal T response, hCG and/or recombinant FSH have been used in some studies (Peng et al. 2022). Adverse effects of all these treatments seem to be mild, but the data is very limited (Shiraishi 2015).

In KS, the testicular effect of aromatase inhibitors (AI) may potentially be beneficial, due to the increased intratesticular aromatase activity in KS (Reifsnyder et al. 2012). The theoretical idea would be to improve the hormonal milieu for spermatogenesis through inhibiting elevated oestradiol levels, which might also increase intratesticular testosterone level (Ramasamy, Ricci, et al. 2009). The effect of preoperative hCG in KS on the serum testosterone level may be more useful prognostically than therapeutically (Guo et al. 2020).

It has been suggested that men with spermatogenic arrest (SA) and hypospermatogenesis might respond to clomiphene citrate treatment prior to MD-

TESE (Aydos et al. 2003; Hussein et al. 2005), or that men with baseline serum FSH below 8 mmol/ml would benefit from FSH pre-treatment (Aydos et al. 2003). The role of medical treatment prior to MD-TESE has been recently discussed in detail in a 2021 review, concluding that the evidence is insufficient to support the use of medical treatment without individual consideration and counselling (Caroppo and Colpi 2021a).

Table 3. Hormonal treatment options of male hypogonadism. (Klami, Mankonen, and Perheentupa 2018)

Hormonal treatment	Dosage
Clomifen citrate (CC)	25–50 mg daily perorally
Tamoxifen	10–20 mg daily perorally
Anastrozole	1–2 mg daily perorally
Letrozole	2,5–5 mg daily perorally
Human chorionic gonadotropin (hCG)	2500–5000 IU 2–3 times a week subcutaneously
Follicle stimulating hormone (FSH)	75–150 IU 2–3 times a week subcutaneously
Human menopausal gonadotropin (hMG)	75–150 IU 2–3 times a week subcutaneously

2.3.3 Surgical method of MD-TESE

The MD-TESE literature is rarely precise in describing the surgical the method in detail, but some consensus exists on the method first described by Schlegel et al in 1999. MD-TESE surgery may be performed using general or local anesthesia. Local anesthesia seems to be more popular in Asian centers (Ishikawa 2012), while many western centers report using general anesthesia. Local anesthesia reduces post-operative pain also in patients operated under general anesthesia (Punjani, Kang, and Schlegel 2021).

Scrotal midline skin incision is made using a scalpel. Usually, the larger testis is chosen for incision through the tunica vaginalis with a scalpel or an energy instrument and the testis is lifted out of the scrotum. The tunica albuginea is then incised with scalpel under an operating microscope. In literature, a transverse incision is sometimes recommended to avoid the equatorial vessels, but no difference in complication rate seems to be associated with vertical incision (Punjani, Kang, and Schlegel 2021).

Mosquito clamps are placed on both sides of the tunical incision to assist in lifting, gently exposing and moving the testis to improve visibility. The aim is to identify the opaquest and dilated seminiferous tubules with the best potential for sperm production (Schlegel 1999). With an operating microscope at 15 to 20-fold

magnification (Esteves 2013), using microsurgical scissors and forceps, the tubules are systematically examined, and the most suitable tubules are removed as biopsies. No difference has been detected whether the biopsies have been taken near the main vessels or not (Schwarzer et al. 2013). When the tissue shows no difference in seminiferous tubule diameter, a mapping technique of taking multiple biopsies systematically of the entire testis is recommended (Beliveau and Turek 2011).

The biopsies are placed on a cell culture plate containing sperm transport buffer and are taken for an immediate laboratory examination by higher magnification. The operation on the larger or the first testis is discontinued after the testis has been thoroughly inspected, or if the laboratory reports finding sufficient amount of sperm for ICSI for at least 3 ICSI attempts. Hemostasis is ensured, the tunica albuginea is closed in running suture using most often 5-0 monofilament suture. The testis is then placed back in the scrotum, the tunica vaginalis is closed using 5-0 monofilament suture and the contralateral testis is operated on in the same manner, if necessary. The skin closure is made with 4-0 absorbable suture in running intracutaneous fashion. Infiltrating spermatic cord local anesthetic after the procedure will reduce the need for post-operative pain medication and is therefore recommended (1% lidocaine + 0.75% bupivacaine, 10 ml for each side) (Punjani, Kang, and Schlegel 2021).

The MD-TESE literature is frequently criticized for the inadequate description of the surgical methods involved. The reporting tends to focus on SRR, while operating time, the equipment used, the complication rates or the laboratory methods may be poorly described. There seems to be a learning curve -phenomenon present, since some studies report improved SRR as well as shorter operating times with longer MD-TESE experience (Ishikawa et al. 2010).

2.3.4 Complications of MD-TESE

In a Japanese study, a drop of 30% was present in circulating testosterone levels of the KS patients after MD-TESE, however returning to the baseline in 12 to 18 months in most men operated (Ishikawa, Yamaguchi, et al. 2009). No significant changes were seen in 46, XY men. MD-TESE leads to less tissue trauma compared to conventional TESE, and results in fewer infections, hematomas, and lower extent of testicular tissue loss (Ramasamy, Yagan, and Schlegel 2005). Testicular pain and minor swelling are common (Esteves 2013). There may be ultrasound changes after MD-TESE, such as hematoma, fibrotic tissue or some calcification. A testicular ultrasound may show signs of fibrosis and calcification, but at six months' follow-up, any changes are seen in only 3–10% of MD-TESE men (Ramasamy, Yagan, and

Schlegel 2005). Overall, the risk of adverse events is small, 3% (Achermann, Pereira, and Esteves 2021).

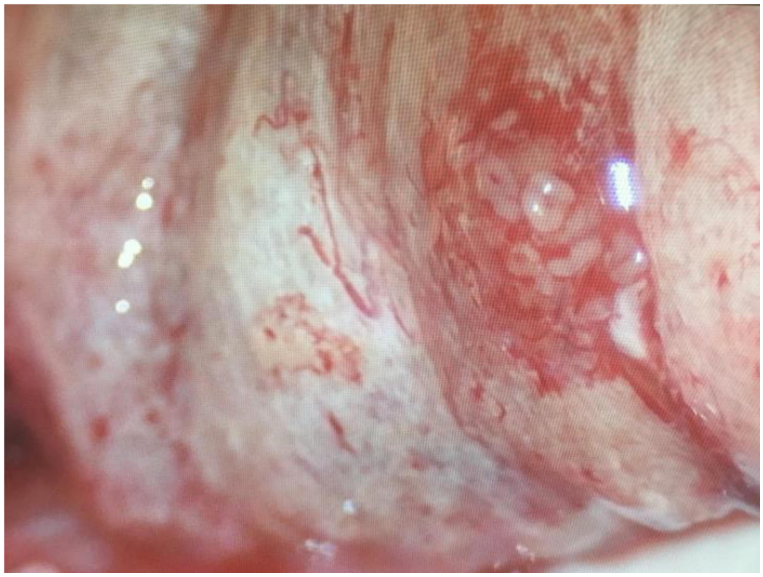

Figure 4. Operating microscope view of a testis of a man with Klinefelter syndrome. Photograph by the author, 2016.

2.3.5 Laboratory analysis of MD-TESE samples

The biopsies from the MD-TESE procedure are transferred to one-well-dishes in 0.5 ml of equilibrated cell culture media. A few biopsies are rapidly dispersed and then quickly screened to inform the surgeon whether any sperm cells are identified in the biopsies. If no sperm cells are found, the embryologist continues by mechanically dispersing the tissue, squeezing the intratubular cell mass out using fine needles (e.g. 27 g) to produce a suspension. Any remaining larger cell clusters are then dispersed by aspirating cell clusters carefully up and down in a 23 g needle. This procedure may be necessary in maturation arrest biopsies, due to the large amount of intratubular cell mass. The use of a collagen digestion to disperse the remaining cell clusters has also been described (Ramasamy et al. 2011), but not found useful in many laboratories (Ishikawa 2012).

After allowing the suspension to settle down for a few minutes, an experienced embryologist starts to thoroughly examine it, using an inverted phase contrast microscope with a minimum of 400-fold magnification. The embryologists and laboratory technicians should be trained to identify different stages of meiotic cells, as the first meiotic prophase cells are easily identifiable and are often visible first in the samples with low sperm cell density. Figure 5a–c.

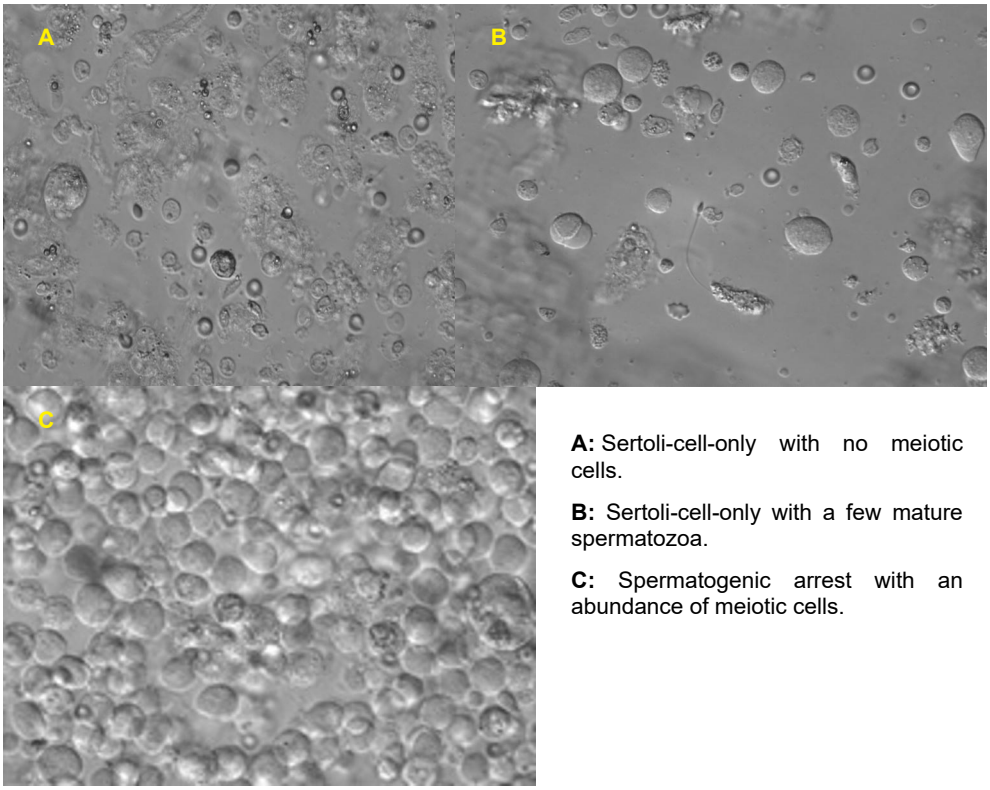

Figure 5. Microscope views of MD-TESE samples. Harri Mankonen, printed with permission.

The samples containing larger amount of sperm cells are pooled and divided into small batches for freezing and future use. The samples with lower number of spermatozoa are pooled and frozen separately from the abundant ones. Freezing, if necessary, is carried out according to the usual sperm freezing protocol of the laboratory, using commercial or home-made freezing media.

The thawing of spermatozoa for the ICSI is carried out according to normal thawing protocol, but minimum number of straws to fertilize the eggs yielded are thawed. Gradient wash is avoided to avoid loss of spermatozoa. Centrifugation wash with equilibrated cell culture media is performed and the supernatant is carefully removed, leaving 50 to 75 µl of media on top of the pellet. Another 50–75 µl of the cell culture media is then added and carefully mixed with the pellet.

Theophylline or pentoxifylline is sometimes recommended to identify viable spermatozoa for ICSI (Ramasamy et al. 2011). The cell suspension is pipetted to form droplets on the ICSI dish, and another droplet is formed using 10 uL polyvinylpyrrolidone (PVP). The suspension droplets are searched for viable sperm cells and transferred to the PVP solution droplet for immobilization. If the number of spermatozoa is insufficient, another straw or ampoule is thawed (Esteves 2013).

2.3.6 Clinical outcome of MD-TESE

2.3.6.1 SRR in MD-TESE

In most men with NOA, there may be small islets containing potential for active sperm production. In OA, needle biopsy techniques such as testicular sperm aspiration (TESA) are successful reaching over 90% SRR (Coward and Mills 2017). There has been an attempt to improve the accuracy of TESA in NOA by introducing mapping techniques and slightly higher SRR has been reached (Beliveau and Turek 2011). Using a needle with larger G may also slightly improve SRR (Carpi et al. 2011).

MD-TESE is 1.5 times more effective in finding sperm than cTESE, stated by a meta-analysis, comparing different methods of sperm retrieval. TESE was found to be twice as likely to find sperm than testicular sperm aspiration (TESA). cTESE seemed to have the highest complication rate, while TESA was cheapest but being often unsuccessful, was rarely the only method used in NOA (Bernie, Mata, et al. 2015).

There is great variation in SRR reported, 18 to 71%, mainly due to patient selection criteria. A large meta-analysis of 4895 patients found that in naïve population, the SRR was 46.8% and 39.1% in patients with previous failed TESA or TESE (Achermann, Pereira, and Esteves 2021). MD-TESE literature is quite justly criticized for several reporting errors. In some studies, SRR results are reported by the number of surgeries, not the number of patients operated. Sperm recovery success rate may also be distorted by including round or elongated, immature spermatozoa in the positive SRR category (Carrell and Simoni 2017). Patient inclusion criteria may at times present another dilemma within the literature surrounding MD-TESE. Only patients with NOA should be included, and other forms of male subfertility, such as cryptozoospermia, severe oligoasthenoteratozoospermia, OA, fertilization failure in ICSI, or hypogonadotropic hypogonadism, should be reported in separate categories.

2.3.6.2 Clinical outcome of MD-TESE-ICSI

There is a lack of uniformity in reporting the MD-TESE-ICSI results, making it difficult to interpret the data on pregnancy results after MD-TESE-ICSI. Some studies report positive pregnancy tests per embryo transfer (ET), others live birth rate (LBR) or cumulative life birth rate (cLBR) per patient. Infertility treatment results as well as medical, economic, and ethical issues surrounding assisted reproductive techniques vary globally. In general, clinical pregnancy rates between 20 and 68% have been reported in MD-TESE-ICSI (Bocca et al. 2017; Ishikawa, Shiotani, et al. 2009; K. Wang et al. 2023). A 2017 meta-analysis on KS patients'

fertility (Corona et al. 2017) found the cumulative pregnancy rate (cPR) and LBR of ICSI to be 50% after MD-TESE.

A 2021 study of 801 embryo transfer cycles compared the pregnancy results of ICSI in patients with NOA or oligoastenoteratozoospermia (OAT) and found no difference between fresh embryo transfer and frozen embryo transfer in terms of pregnancy results in couples with NOA and OAT as male infertility factor (Sik et al. 2021). The methods of sperm recovery varied, and the reassuring results cannot thus be directly extrapolated to MD-TESE-ICSI.

In some men, the most mature form of spermatozoa found are round or elongated spermatids. They were considered ineffective in fertilizing oocytes, until a 2016 Japanese study reported a fertilization rate of 21% and the birth of 14 children after round spermatid injection (Tanaka et al. 2015). The clinical success rates are low, and more data is needed, but the use of round spermatids may have future potential as a treatment option for some men with SA diagnosis (Tekayev and Vuruskan 2021).

The use of frozen or fresh MD-TESE sperm for ICSI is still a matter of debate. A synchronized ovarian stimulation is needed for the fresh sperm MD-TESE-ICSI, which is the most rational reasoning behind freezing, given the fact that generally viable sperm are found in MD-TESE of less than half of NOA men. Some centres report a failure to find sperm in up to one third of the frozen samples (Schlegel et al. 2004; Z. Zhang, Jing, et al. 2021), while many other centres report no such issues with freezing and thawing (Tavukcuoglu et al. 2013) (Almekaty et al. 2019; Corona et al. 2019). The benefits of freezing also include easy transport, no need for donor sperm back-up and potential for fertility preservation.

In a Belgian study, a cumulative live birth rate of only 13.5% was reported after sperm recovery by conventional TESE and following ICSI treatments for NOA, mainly due to patient drop out (Vloeberghs et al. 2015). This has led to some concern about counselling as well as the need for psychological support for the patients. In a 2018 Egyptian study, 141 MD-TESE-ICSI-cycles and 23 frozen embryo cycles were assessed, leading to 62 pregnancies and 37 live births. The only factor significantly affecting the result was the female age, whereas the aetiology of NOA or the histopathology had no effect on the LBR (Almekaty et al. 2019). In a 2021 study of 130 micro-TESE-ICSI cycles, a fertilization rate of 85%, clinical pregnancy rate of 22.3% and LBR of 11% was observed, with no significant differences between different aetiological groups (Vahidi et al. 2021). A meta-analysis from 2021 reported live birth in 24% and clinical pregnancy in 39% of the patients who had an embryo transfer after MD-TESE-ICSI (Achermann, Pereira, and Esteves 2021). In a study of comparing 40 fresh versus 30 cryopreserved spermatozoa micro-TESE-ICSI outcomes, both groups showed high fertilization rates 55 and 53% (NS), implantation rates 65 and 60% (NS) and live birth rates of 75 and 50% (P=0.04), favouring fresh sperm cycles (Z. Zhang, Jing, et al. 2021).

Very few data are available on the effect of the different causes of NOA on the ICSI outcome. In a Chinese study of 347 micro-TESE-ICSI-cycles, the fertilization rates per oocyte were 45 to 65%, but among men with Y deletion only 27%. This also led to a low clinical pregnancy rate of 20% while the other diagnoses led to CPR of approx. 50% and previous orchitis group CPR of 78%. There was no difference in miscarriage rates or foetal abnormalities (H.L. Zhang, Zhao, et al. 2021).

The initial reports on the health of the offspring after ICSI with testicular sperm are reassuring (Meijerink et al. 2016; Tsai et al. 2015; Mantravadi, Rao, and Sree 2022; Fedder et al. 2013), but conclusive data on children born after MD-TESE-ICSI are still lacking. Analysis of the sperm haplotype in men with KS has shown that the sperm contain a normal chromosomal composition, making the initial concerns about the potentially increased risk of chromosomal abnormalities in offspring unnecessary (Madureira et al. 2014). Y chromosome microdeletion is, however, directly passed on to all male children of males with this abnormality. It is therefore essential to offer genetic counselling to the men carrying Y chromosome microdeletions prior to any fertility treatments (Krausz and Chianese 2014).

In idiopathic NOA, a genetic unidentified abnormality is thought to be present in a high proportion of men (Miyamoto et al. 2017). As these abnormalities may be passed on to the male offspring, it is possible that the male child presents with the same form of NOA as the father. It is also not conclusively known whether the use of an immature gamete, that has not yet gone through all the epigenetic processes and maturation in the epididymis, affects the health of the offspring or the generations to follow.

2.4 Factors predicting MD-TESE success

2.4.1 Clinical findings

In healthy population of men, testicular volume (TV) is associated with spermatogenetic function of the testis (Lenz et al. 1993). In azoospermia, however, it mainly helps to distinguish between OA and NOA, as described earlier. In NOA, testicular size seems to have limited value in predicting SRR in MD-TESE (H. Li et al. 2017; Bernie, Ramasamy, and Schlegel 2013). Men with KS generally have very small testicular size and very good SRR, which may partly be responsible for this finding, and therefore in studies excluding KS, testicular size has been shown to predict better SRR outcome (Kizilkan et al. 2019). A meta-analysis combining MD-TESE and cTESE SRR results of men with NOA showed an increase of SRR with increasing testicular volume (Corona et al. 2019).

Although men are usually considered to remain fertile throughout their lifetime, several fertility issues have been associated with advanced paternal age. Higher

sperm DNA fragmentation, prolonged time to pregnancy and increased miscarriage rate have been linked to advanced paternal age. Several childhood illnesses have also been shown to increase with advancing paternal age (Nybo Andersen and Urhoj 2017; Ramasamy et al. 2015). MD-TESE results seem to remain very good at an older age (Ramasamy and Schlegel 2014), although some centres report declining results for older men (Aljubran et al. 2022). In KS, however, the SRR declines with age, but with good overall prognosis associated with KS, male age rarely presents a reason to decline MD-TESE (Okada et al. 2005).

Obese men have very similar sperm recovery results in MD-TESE compared to lean men. No differences are seen in SRR results of normal weight and obese KS men, either (Kort et al. 2006; Iwatsuki et al. 2017). Obesity and elevated BMI of the male partner are however associated with higher sperm DNA fragmentation index, unfavourable hormonal profile (high FSH, LH and low T) and reduced spontaneous fertility (Kort et al. 2006). There is some data reflecting worse ICSI results using obese men's MD-TESE sperm, but conclusive data is missing on the pregnancy outcome (Ramasamy et al. 2013).

Varicocele is common finding in fertile and subfertile men, affecting 5% of men with NOA (Esteves et al. 2016). Although varicocele is associated with male subfertility (Damsgaard et al. 2016), its complete pathophysiology or role in NOA is not fully understood (Hamada, Esteves, and Agarwal 2013; Agarwal, Hamada, and Esteves 2012). Some results indicate that NOA men may benefit from microsurgical varicocelectomy prior to MD-TESE in terms of an improved SRR, but the time interval between the two or the effect on pregnancy results are not known (Esteves et al. 2016).

2.4.2 Serum hormone levels

As serum follicle stimulating hormone (FSH) level predicts testicular spermatogenetic function quite well (Bergmann, Behre, and Nieschlag 1994), it is also of assistance in making the differential diagnosis of NOA vs. OA (Madani et al. 2012). However, there is no conclusive data showing, that high FSH would accurately predict poor outcome of MD-TESE. In some studies, a higher FSH has been associated with a lower SRR in MD-TESE (Aljubran et al. 2022; Kizilkan et al. 2019) but most data suggest that FSH is a poor predictor of MD-TESE success. In his 2012 meta-analysis, Madani compared the serum FSH levels in a total of 1261 men with NOA with their MD-TESE success, showing FSH to display a low predictive value for SRR(Madani et al. 2012).

Serum testosterone (T) level at baseline is a very poor predictor of sperm recovery in MD-TESE (Althakafi et al. 2017). However, testicular ability to respond to hCG may have some prognostic value, especially in KS men (Guo et al. 2020). Higher serum inhibin B level as a single variable does not predict SRR reliably but

may in the future be useful as a part of a predicting model (Cissen et al. 2016). Anti-Müllerian hormone (AMH) is a functional marker of foetal Sertoli cells and higher AMH has been suggested to be helpful in predicting in MD-TESE in men with idiopathic NOA (Pozzi et al. 2023). Combining AMH and inhibin B has also shown promise in predicting SRR in NOA (Deng et al. 2023).

Multivariate prediction models and machine learning models have been created to predict SRR in NOA more precisely. Most of them have been created by combining several clinical factors, such as the NOA diagnosis, the male age, BMI, TV, ethnicity, and semen volume, with hormonal status (T, FSH, LH, leptin, AMH) (Cissen et al. 2016; Zeadna et al. 2020; Chen et al. 2022). Some prediction models combine different seminal plasma biomarkers, with or without the clinical and hormonal data described (Xie et al. 2020; Ji et al. 2021). Artificial neural networks (ANNs) are proving to be of assistance in setting up these models (Ma et al. 2011; Zheng et al. 2023). A few of these are presented in table 4.

Table 4. Receiver operating characteristics (ROC) curve analysis, sensitivity and specificity for predicting sperm recovery (SR) in non-obstructive azoospermia (NOA) with various parameters. FSH=follicle stimulating hormone, LH=luteinizing hormone, AMH=anti müllerian hormone, T=testosterone, TV=testicular volume, BMI=body mass index, AUC=area under the curve

Author, year, journal	N	Inclusion criteria	Parametres used	Outcome
Ma et al, 2011, Hum Reprod	280	NOA, MD-TESE	TV, serum leptin, FSH, LH, T and PRL, seminal plasma leptin, seminal plasma volume, SR	AUC 0.83
Cissen et al, 2016, Hum Reprod	1371	NOA, cTESE	Age, diagnosis, serum T, FSH, LH, SR	AUC 0.69
Zeadna et al, 2020, Hum Reprod	119	NOA, cTESE	machine learning model, FSH, LH, T, semen volume, age, BMI, ethnicity, TV, SR	AUC 0.8
Xie et al, 2020, Hum Reprod	96	idiopathic NOA, MD-TESE	lncRNA-panel*, SR	AUC 0.986 with score <0.532
Chen et al, 2022, Asian J Androl	162	cryptorchidism, NOA, MD-TESE	bi- vs. unilateral orchidopexy, location of testis, age at orchidopexy, SR	AUC 0.907
Shi et al, 2023, Front Endocrinol	114	idiopathic NOA, MD-TESE	circRNAs, s-FSH, LH, T, SR	AUC 0.857
Zheng et al, 2023, Reprod Sci	261	idiopathic NOA, MD-TESE	s-AMH, SR	Sensitivity 0.870, spesificity 0.705

* LOC100505685, SPATA42, CCDC37-DT, GABRG3-AS1, LOC440934, LOC101929088 (XR_927561.2), LOC101929088 (XR_001745218.1), LINC00343 and LINC00301

** circ_MGLL, pathological circRNA types

2.4.3 Aetiology of azoospermia

Aetiology of azoospermia is one of the few factors contributing to reliable prediction of SRR in MD-TESE (H.L. Zhang, Zhao, et al. 2021). However up to 60–70% of NOA is idiopathic, with no known cause for the condition (Cissen et al. 2016; Bernie, Mata, et al. 2015). These men have 30–40% chance of sperm recovery in MD-TESE, which is quite low SRR compared to overall results, but still much higher than with any other method of sperm recovery (Deruyver, Vanderschueren, and Van der Aa 2014; Achermann, Pereira, and Esteves 2021).

Klinefelter syndrome is a chromosomal abnormality with an additional X chromosome, occurring in approximately 1–2 per 1000 live male births. KS is one of the diagnoses with the highest SRR in MD-TESE. Very small testicular volume and intratesticular changes, including progressive tubular sclerosis, make their TESA procedure difficult and unsuccessful. However, in MD-TESE, it is easy to visualize the remaining seminiferous tubules with spermatogenesis. The SRR varies between 50 to 70% (Dabaja and Schlegel 2013), and the results have shown a tendency to worsen with advancing age in some studies (Emre Bakircioglu et al. 2006). Most expert opinion references do not recommend sperm retrieval and fertility preservation attempts for teenagers, since the results are very reassuring through young adulthood (Gies et al. 2016).

Y chromosome microdeletion is a group of disorders caused by deletions within the male-specific region of the Y chromosome. Y chromosome microdeletion is known to be present in 13% of NOA cases (Reijo et al. 1996). The deletion may be in the proximal (AZFa), the middle (AZFb) or the distal (AZFc) segment of the Y-chromosome, the latter being the most common. Y chromosome microdeletion of AZFc region is associated with the most favourable SRR of 60 to 70%, while SRR in Y chromosome deletions in AZFa, AZFb regions, or a combination of these, is consistent with very poor SRR results, and most centres will refrain from MD-TESE in presence of these diagnoses (Krausz and Chianese 2014).

Cryptorchidism or undescended testes is associated with and increased risk of testicular malignancy as well as reduced fertility (Radmayr et al. 2016). In MD-TESE, the men with previous cryptorchidism generally have a high success rate, 64 to 82% (Bernie, Ramasamy, and Schlegel 2013). The timing of the corrective surgery has been shown to affect future fertility, but the effect on MD-TESE results has not been shown (Rohayem et al. 2017; Radmayr et al. 2016).

It is very difficult to accurately predict sperm retrieval after previous exposure to radiation and cytotoxic agents. The main reason is the heterogeneity of the diseases treated, as well as the amount of radiation and different target organs, and the variety of cytotoxic agents used. In the studies published to date, these men usually have 50 to 60% SRR (Shin et al. 2016). In a recent report, 106 men with previous radiation and/or cytotoxic medication had MD-TESE, with an overall SRR

of 37%. The group with only cytotoxic medication without radiotherapy had a higher chance of sperm retrieval compared to the men with radiotherapy, and none of the men with previous pelvic radiation had spermatogenesis (Brant et al. 2022). Sperm cryopreservation should always be offered as the most effective method for fertility preservation, but more information on other strategies is needed. The ongoing research projects surrounding fertility preservation in male childhood malignancies will probably bring more light into this topic (Ho et al. 2017).

2.4.4 Histopathological findings

It is possible to clinically reach an accurate NOA diagnosis in nearly 90% of patients (Shin et al. 2016). Many patients however may have had a needle biopsy procedure prior to MD-TESE, giving knowledge of the histopathological finding. A previous unsuccessful TESE or TESA may worsen the expected SRR in MD-TESE, but the effect is not clinically significant enough to refrain from performing the MD-TESE (Ramasamy and Schlegel 2007).

Histopathological diagnosis is a predictive factor for SRR prior to MD-TESE (Gies et al. 2016; Aydin et al. 2015; Bernie, Ramasamy, and Schlegel 2013). One of the most common findings is Sertoli-cell-only (SCO), which is associated with a SRR below average in MD-TESE. In SCO, SRR of 30% has been reported. In spermatogenic or maturation arrest (SA), slightly higher rates of 40 to 60% are reported (Bernie, Shah, et al. 2015). SA is classified as early and late maturation arrest, and in early maturation arrest sperm is thought to be present in half as many patients undergoing MD-TESE compared with late maturation arrest, with the SRR of 40% and 78%, respectively(Bernie, Shah, et al. 2015). SA can also be classified as local or diffuse, with significantly lower SRR in diffuse vs. local SA (Bernie, Shah, et al. 2015).

2.4.5 Seminal plasma biomarkers in predicting MD-TESE success

Conventional evaluation of a patient with azoospermia, as described in page 20 is often inadequate to reliably make the differential diagnosis between OA and NOA, and rarely assists in predicting sperm recovery in MD-TESE. A variety of biomarkers present in the seminal plasma of males with azoospermia have been found to give promise as novel ways to guide in more accurate preoperative counselling of these men.

Seminal plasma was historically seen as an inactive medium with no other function but to carry the sperm cells. This concept has been proven false as seminal plasma is a complex fluid comprising of secretions from several parts of the seminal

tract and from the seminiferous tubules in the testicles, also to protect and nourish spermatozoa after ejaculation up to fertilization and act as a functional modulator of spermatozoa function. Seminal plasma contributes to activating the endometrial gene expression and immune cell changes at fertilization to ensure healthy implantation (Robertson and Sharkey 2016).

The most studied seminal plasma proteins linked to the presence of spermatogenesis include seminal prostaglandin D synthase (L-PGDS), acrosome protein SP-10 (ACRV1), soluble lectin galactoside binding protein 3 (LGALS3BP), extracellular matrix protein 1 (ECM1), and testicular tissue monospecific protein TEX101 (Araujo and Bertolla 2021). Utilizing ECM1 and TEX101 may be useful in the diagnosis of NOA vs. OA but also in predicting TESE outcome. LGALS3BP level less than 153 ng/ml was associated with reduced chance of sperm recovery in cTESE (Freour et al. 2013). Gashti et al 2022 studied the seminal plasma of 48 men undergoing micro-TESE and found that seminal phosphoglycerate kinase 2 (PGK2) concentration of 136.3 pg/ml and acrosin (ACR) concentration of 21.75 mIU/ml could be used as cut-off values for the prediction of MD-TESE-ICSI outcomes in NOA patients(Gashti 2022).

Seminal plasma AMH, leptin and inhibin B have not been found useful in predicting sperm recovery success in cTESE and MD-TESE (J. Li et al. 2023), although leptin has more potential combined with other markers (Ma et al. 2011). ESX1 mRNA expression in the testis has been suggested to correlate with sperm recovery success in cTESE and MD-TESE (Pansa et al. 2014).

MicroRNAs (miRNAs) are family of small, noncoding single stranded RNA molecules of 18 to 25 nucleotides. They are negative regulators of messenger RNA transcription and translation. MiRNAs have been detected in human spermatozoa and in seminal plasma, and they are regarded one of the most important regulators of spermatogenesis (Ostermeier et al. 2002; C. Wang et al. 2011). Abnormal expression of miRNAs has been linked to OAT (Abu-Halima et al. 2016). MiRNA expression profiles in different histopathological subtypes of NOA show great variation (W. Zhang, Zhang, et al. 2021). It is expected, that due to their important role in spermatogenesis and to the abnormal expression of many miRNAs in with NOA, miRNAs may have potential in predicting MD-TESE success. Zhang et al studied a miRNA panel for 76 men with NOA, 53 with positive and 23 with negative sperm retrieval. Their predictive model reached a good accuracy (AUC = 0.927) (Y. Zhang et al. 2022).

Long non-coding RNAs (lncRNAs) are a group of RNA transcripts with longer than 200 nucleotides with no protein-coding capacity. Since lncRNAs also have a big role in spermatogenesis (A.C. Luk et al. 2014; Joshi and Rajender 2020), they may have potential to predict SRR. Xie et al created and validated a prediction model to predict SRR success in MD-TESE by using 9 lncRNAs expressed in the testis.

These prediction models have great potential (table 2), but larger studies are needed to confirm their diagnostic value in future clinical practice (Xie et al. 2020).

2.4.6 Imaging in predicting MD-TESE success

Testicular ultrasound is recommended for all men with azoospermia, as it is an inexpensive, safe, and noninvasive imaging method. In Finland, ultrasound is considered an office imaging method for gynecologists and many urologists, making it widely accessible. It is however very rare, that a specific NOA diagnosis can be reached with a scrotal ultrasound, and it is useful mostly in ruling out neoplasm or other conditions such as varicocele. Doppler ultrasound has been studied but its added value in evaluating the presence of spermatogenesis has not been very convincing (Tunç et al. 2005). Contrast enhanced ultrasound (CEUS) has been studied in NOA to identify the testicular areas with best perfusion prior to MD-TESE, and with the method, a SRR of 65.8% was reached, when in the control group the SRR was 47.5% ($p>0.05$) (S. Zhang et al. 2018).

There are not many studies that combine magnetic resonance imaging (MRI) to be a part of andrological examination of male infertility or azoospermia. Many novel applications of MRI have arisen with diffusion-weighted imaging (DWI), a form of MRI that is based on the movement of water molecules in tissue. Apparent diffusion coefficient (ADC) measurement is used to create a numerical vale to the diffusion restriction of a particular tissue – a method widely used in central nervous system malignancies as well as the diagnosis of stroke. Magnetic transfer ratio (MTR) is another quantitative MRI method to measure the macromolecular content of the tissue studied (Tsili et al. 2017).

Increased testicular ADC has been associated with lower spermatogenetic potential when men with NOA and healthy control subjects have been compared (H. Wang et al. 2018; Tsili et al. 2018). ADC, MTR and the volume of the testis have also been studied as potential markers to help identify NOA and OA (Han et al. 2018; Regent et al. 2020; Cai et al. 2021). Ntorkou et al found that reduced testicular volume in MRI correlated negatively with success in MD-TESE, while ADC and MTR were both increased if the sperm retrieval was not successful in MD-TESE, however, there was a great overlap in the numerical values between SRR negative and positive groups (Ntorkou et al. 2019).

3 Aims

1. The first aim of this study was to thoroughly assess the SRR and complications of the MD-TESE at the Turku University Hospital. This was necessary to validate our material for further studies.

2. The second aim was to add to the literature to assess the overall efficacy and safety of MD-TESE-ICSI, being a novel treatment option for the most severely infertile men.

3. More than half of the men selected for MD-TESE will end up having no sperm recovered and frozen, and we are currently unable to identify the NOA men with active spermatogenesis. The third aim was to find ways to better predict the MD-TESE outcome by recording detailed information on the men attending the study, and by performing testicular MRI and measuring their ADC values.

4 Materials and Methods

4.1 Study Populations and Study Designs

4.1.1 Study I

For the first part of this study, the first consecutive 100 men operated on in years 2008 to 2015 were analyzed retrospectively. Repeated semen analyses using the WHO criteria of that time were performed to verify azoospermia. NOA was diagnosed by endocrine and genetic analysis. Y chromosome microdeletions, karyotype, serum concentrations of FSH, LH, PRL, testosterone and thyroid stimulating hormone were assessed. Testicular ultrasound and a physical examination were performed prior to MD-TESE.

The men presenting with low testosterone were treated with aromatase inhibitor (AI, n = 26), tamoxifen (n = 11), human chorionic gonadotropin (hCG, n = 6) or clomiphene citrate (CC, n = 5), started 4 to 6 months prior to MD-TESE to reach normal serum testosterone levels. AI was chosen for the KS men if they were able to tolerate the treatment. 48% of the men operated on received medical treatment preoperatively. The average pretreatment testosterone level was 7.4 nmol/L and post-treatment level 22.1 nmol/L. Complete data were available in 35 men treated medically.

We also assessed the ICSI treatments performed using the frozen MD-TESE sperm, including the treatments known to us by 2015. At that time, 32 couples had gone through at least one cycle of ICSI. The straws of cryopreserved testicular tissue were transferred to ten fertility units within Finland. The ICSI treatments were conducted using different stimulation protocols. Other factors affecting the ICSI outcome were not recorded, for example the age, weight, and potential diagnoses of the female partner. There may have been large variation in these parameters.

4.1.2 Study II

The data collection was retrospective. The study protocol to collect and analyse the data from Turku University Hospital Babe database (Babe 3.3b IVF Management system 1996–2023, Cleodora Medical Software Lda., Trafore OY Helsinki Finland)

from 2011 to June 2023 received approval from the clinical research centre of the University of Turku and the Hospital District of Southwest Finland. No ethical committee approval was required for collecting and analysing these data retrospectively.

During the selected period, there were 71 MD-TESE-ICSI treatments in our hospital (group microTESE). They were all included. We wanted to control the treatment results using other ICSI treatments for male infertility as controls. The first control group (OAT group) consisted of ICSI treatments with male factor infertility (oligozoospermia with sperm concentration less than 15 million per millilitre, with or without astenozoospermia and/or teratozoospermia, n = 340). For the second control group, ICSI treatments with mainly frozen sperm collected by testicular needle biopsy (TESA) were selected (TESA group N = 52). These men had obstructive azoospermia (OA) due to history of trauma, infection, surgical trauma, hereditary causes, or unexplained OA. We did not perform TESA for men who had been previously vasectomised. Our policy was to promote and demand smoking cessation, so to our knowledge all the men in the groups were non-smokers.

Babe software was used to search for information on the ICSI treatments carried out at Turku University Hospital. The data was collected with the "sperm origin" as the search tool annotated to the database. We also collected the age and the body mass index (BMI) of the men and the women treated. Treatment protocol, mature egg yield, fertilization rate and IVF complications were investigated. We collected the data on fresh and frozen embryo transfers. Pregnancy rate, clinical pregnancies, pregnancy loss, live birth rate, birth weight, twinning rate, delivery route and pregnancy complications were studied.

4.1.3 Study III

The study design was prospective. We included men with NOA who were to be treated with MD-TESE surgery in Turku University Hospital. The study population men (n = 21) were operated between 2016 and 2020. We were aiming to differentiate the men with positive sperm recovery result (n = 10) from the men with no sperm recovered (n = 11) with diffusion-weighted imaging. Positive sperm recovery was defined by the presence of mature sperm in biopsies. We included a fertile control group (n = 9) recruited from secondary infertility couples at our unit, with inclusion criteria of normal sperm analysis according to WHO 2010 criteria and at least one spontaneously conceived child.

4.2 Surgical methods, ovarian stimulation and ICSI

4.2.1 The surgical method of MD-TESE

In 2008-2020, the patients were given general anaesthesia to perform MD-TESE. From the beginning of 2020, all the men were operated in local anaesthesia, using 1% lidocaine + 0.75% bupivacaine, infiltrating 10 to 15 ml for each side and 2 to 5 ml for the scrotal skin incision.

The skin was incised in the scrotal midline with a scalpel, and the tunica albuginea was vertically opened with a scalpel in most cases. We used an operating microscope wit 20-fold magnification to target the biopsies at the most eligible tubules, opaque and largest in diameter. The biopsies were picked up by the IVF laboratory staff for examination by 400-fold magnification. Microsurgical forceps and scissors were used to remove the tubules for biopsies, which were placed in four-well cell culture plates containing sperm transport buffer (G-Gamete^{TM}, Vitrolife Sweden AB, Frölunda, Sweden). We took 15 to 30 biopsies, until a sufficient number of spermatozoa was recovered for the purposes of one to three ICSI treatments. In cases with no spermatozoa present, we discontinued when the testicular circulation was compromised, or when the testicular tissue had been completely mapped. A biopsy was also sent for histopathology. Hemostasis was ensured using bipolar electrocauterization, and the tunica albuginea was closed in running 5-0 monofilament suture. The testis was placed back in the scrotum, the tunica vaginalis was closed using 5-0 monofilament suture and the contralateral testis was operated on in the same manner, if necessary. The skin was closed with 4-0 absorbable running intracutaneous suture. Local anesthetic was infiltrated at this stage for the men under general anesthesia.

The female partners' ICSI treatments were not synchronized in any of our study protocols; all testicular tissue with positive sperm recovery was cryopreserved.

4.2.2 The laboratory method of MD-TESE

The extracted testicular tissue was mechanically dispersed, and the cell suspension was thoroughly examined by experienced laboratory staff using a microscope with 400-fold magnification microscope. If no sperm was found, a collagenase digestion was performed, and the tissue was re-examined. As the initial 100 operations showed no benefit from collagen digestion after unsuccessful manual dispersion, we discontinued this practice.

Samples containing a large number of sperm cells were pooled and divided into small batches for freezing. The samples with low number of spermatozoa were pooled and frozen separately. Regardless of the quality and the number of observed

spermatozoa, the samples were divided into at least ten straws or ampoules (CBS™ High Security sperm straw 0.5ml, CryoBio System, Saint Ouen Sur Iton, France). Cryopreservation was done according to the usual sperm freezing protocol using a commercial freezing media (SpermFreeze Solution™, Vitrolife Sweden AB, Frölunda, Sweden).

4.2.3 TESA

Testicular sperm aspiration (TESA) in Study II was performed in local anaesthesia, with spermatic cord block and skin anaesthesia combined with intravenous alfentanil analgesia and at times with midazolam sedation, if necessary. A small skin incision was made, and a 14G Biopty™ -biopsy punch gun (Bard Urological, Covington, GA, USA) was used to take up to six samples. The testis was manually compressed to avoid hematoma or swelling, while the biopsies were microscopically screened to ensure sufficient number of sperm. The sperm biopsies were mostly frozen to be thawed in future ICSI treatments, but in some cases coordinated ICSI cycles were performed, and only surplus sperm were frozen according to the standard sperm freezing protocol (SpermFreeze Solution™, Vitrolife Sweden AB, Frölunda, Sweden).

4.2.4 Preparing the semen samples for ICSI

A straw containing frozen micro-TESE sperm of was thawed on the day of the egg retrieval of the female partner, using a standard sperm thawing protocol of the laboratory. The loss of spermatozoa was avoided by refraining from gradient wash, as the sperm number was very low in many cases. A 2000 to 2500 x g short centrifugation wash was however performed to separate a pellet to be mixed with cell culture media (G-IVF PLUS™ Vitrolife Sweden AB, Frölunda) to reach 150µl volume of cell suspension. Theophylline (GM501 SpermMobil™, Gynemed GmbH&co, Lensahn, Germany) was often needed to reliably identify viable sperm cells to be chosen for ICSI.

Long, thin droplets of this cell suspension were formed on the ICSI dish and covered in oil. Each droplet was thoroughly examined to collect any viable sperm cells, which were placed in a polyvinolpyrrolidone (PVP, ICSI™, Vitrolife Sweden AB, Frölunda, Sweden) solution droplet on the same dish, to immobilize the sperm for ICSI. After searching through all the droplets of cell suspension, the number of sperm cells was estimated, and another straw was thawed in the event of insufficient sperm yield for ICSI. A standard ICSI procedure was then performed, as described by Palermo in 1992 (Palermo et al. 1992).

On the oocyte retrieval day of the ICSI cycle with ejaculated sperm, the samples were processed with a gradient wash according to the standard protocol described by the manufacturer (PureSpermTM, Nidacon International, Mölndal, Sweden). The samples containing very few sperm were not processed with a gradient wash to avoid the loss of sperm but processed in the same manner as the thawed micro-TESE and TESA samples.

4.3 MRI and image analysis

In the TESE-MRI study, the same magnetic resonance scanner was used to acquire the MRI images (Ingenia 3.0 T, Philips, Best, The Netherlands). The study subjects were in prone position while the scanning of the pelvis took place, using a 32-channel coil for torso. T2 weighted images were acquired in three planes. Diffusion weighted imaging with low b-values (0, 100, 200, 300, 350 and 500), medium b-values (0 and 1500) and high b-values (0 and 2000) were included in the protocol. The MR parameters are shown in detail in Table 5.

Table 5. Imaging parameters. Modified from original publication III, reprinted with permission. DWI=diffusion weighted imaging, T2W TSE=T2 weighted turbo-spin echo, EPI= echo-planar imaging

Parameter	T2W TSE	T2W TSE	T2W TSE	DWI	DWI	DWI
Imaging Plane	Sagittal	Axial	Coronal	Axial	Axial	Axial
Repetition time (ms)	4844	4375	3772	4723	3180	3105
Echo time (ms)	95	95	90	46	56	60
Flip angle (°)	90	90	90	90	90	90
Number of slices	42	44	36	23	23	23
b-value, 10^6s/mm2	-	-	-	0, 100, 200, 350, 500	0, 1500	0, 2000
TSE/EPI factor	28	17	19	23	14	14
Section thickness (mm)	3	3	3	3	5	5
Acquisition matrix size	316 x 237	260 x 243	260 x 218	124 x 124	124 x 124	124 x 124
Field of view (mm)	220 x 220 x 138	220 x 145 x 220	119 x 220 x 220	250 x 69 x 250	250 x 70 x 250	250 x 70 x 25

ADC maps with regions of interest (ROIs) were drawn. Horos DICOM viewer version 4.0.0RC4 (Horos Project, GNU General Public Licence version 3.0, San Diego, CA, USA) was used to analyse these charts. Three central slices of the testicle

were measured, avoiding the tissue borders to avoid partial volume effect of large 5 mm voxels. The ROI border on the image was placed inside the testicle, a few mm from the tissue border. The ROI size thus depended on the testicular volume. 6 measurements were made of each study subject on each ADC map.

4.4 Statistical analysis

4.4.1 Study I

The study analysing the first 100 men used SAS for Windows version 9.4 (SAS Institute, Cary, NC, USA) for data analysis. The sperm recovery rate (SRR) was analysed using a logistic regression analysis, comparing SRR in relation to different categorical variables, such as medical treatment prior to the operation, normal vs. small testicular size and whether a previous biopsy had been performed or not. The relation between the patient age and successful sperm recovery was analysed by an independent sample t-test. The serum hormone concentrations in relation to surgical sperm retrieval success were compared by Mann–Whitney U-test. 0.05 was considered statistically significant. The numbers were too small to show statistical significance, but some tendencies were observed.

4.4.3 Study II

For the severe male factor ICSI treatments studied, the statistical analyses were performed using JMP® Pro 17.0.0 for Macintosh (JMP Statistical Discovery LLC, Cary, NC, USA 27513). Data were expressed in mean (± SD) or percentages. The comparisons between the study groups used non-parametrical tests and P values < 0.05 were considered statistically significant.

4.4.2 Study III

For the magnetic resonance imaging study, the statistical analysis was performed using JMP Pro statistical software version 16.2.0 (SAS Institute Inc. and JMP Statistical Discovery LLC, Cary, NC, USA 27513). The p-values less than 0.05 were considered statistically significant. A Shapiro-Wilk W test was used to analyse each dataset for normal distribution. Normally distributed numerical data were presented as a mean ± standard deviation and data with a skewed distribution were presented as the median [interquartile range (IQR)]. Normally distributed data were compared with the Tukey–Kramer test for all pairs and data with skewed distribution were compared with a nonparametric Steel–Dwass method for all pairs.

4.5 Study ethics

Studies I and II were approved by the hospital district but due to the retrospective nature did not require an ethical council approval or subject consent. Study III was approved by the common ethical council of the university of Turku and the Hospital District of Southwest Finland, and an informed consent was obtained from our study participants.

5 Results

5.1 Study I population characteristics

To assess the clinical outcome of MD-TESE, we assessed the first 100 men operated at our clinic. 50% of the men had been performed a previous needle aspiration biopsy. No repeated MD-TESE operations were included. The needle biopsies had been performed in different referring clinics using various methods, and we only included men with previous negative biopsies in this study, or to be operated with MD-TESE. The histopathological diagnosis of the previous biopsies was gathered. Sertoli-cell-only was the most prevalent histological finding, present in nearly all previously biopsied men, indicating the lack of spermatogenesis in these biopsies (n = 41). The remaining men had spermatogenic arrest diagnoses (n = 9).

The average age of the study men on the operation day was 33.4 years, ranging between 21 and 47. The testicular size was measured by an orchidometer at surgery, and defined as small, or less than 15 ml, in 56% of the men. The men with Klinefelter syndrome (KS) had a testicular size less than 5 ml, whereas all the men with spermatogenic arrest histopathology had a testicular size more than 15 ml. Mean testicular size of the men in study I was 13 ml and median testicular size was 12 ml.

The aetiology of NOA was KS in 15% and Y-deletion AFZc in 7% of the men. 10% had previous cryptorchidism or torsion, and 3% had been treated with cytotoxic medication or radiation. In large majority (65%) of the men, there was no known diagnosis or cause for NOA. The study I population is shown in Table 6.

Table 6. Study I population (Adapted from study I). FSH = follicle stimulating hormone, LH = luteinizing hormone, SR = sperm recovery.

	ALL (n = 100)	SR positive (n = 42)	SR negative (n = 58)	p-value SR positive vs. negative
Age at surgery (years)	33.4	32.9	33.7	0.496
Testicular size <15 mL	54	29	25	0.339
Serum testosterone (nmol/L)	16.2	16.9 (5.5–33.0)	15.5 (4.0–31.0)	0.322
Serum FSH (U/L)	23.1	21.5 (4.0–50.0)	24.2 (4.2–74)	0.488
Serum LH (U/L)	11.5	9.7 (2.1–30.0)	10.8 (3.0–35.0)	0.436
Previous biopsy	48	22	26	0.456

5.2 MD-TESE surgical outcome (Study I)

In Study I, sperm was retrieved and frozen from 42% of the men with NOA by MD-TESE. Sperm recovery rate (SRR) was 31% in men with unexplained NOA, which was the largest etiological group (n = 65). In men with KS, the SRR was 40% (n = 15), 90% in men with previous cryptorchidism or torsion (n = 10), 57% in men with Y deletion AFZc (n = 7) and 67% in men with a history or cytotoxic treatment or radiation (n = 3) (NS).

In study I, SRR seemed higher in men with small testes (less than 15 ml) compared to the men with normal of large (more than 15 ml) size testes (46 vs. 36%; p = 0.339, NS). Younger men did not have more favorable prognosis overall compared to older men, as the mean age in the SR+ group was 32.9 and the mean age of the SR- was 33.7 years (p = 0.496, NS). There was vast variation in preoperative, unmedicated serum FSH level, but we found no predictive value connected to the elevated FSH, as the group with positive SR had mean FSH of 21.5 IU/L, while the group with negative SR had mean FSH 24.2IU/L (p = 0.488, NS).

50% of the study subjects had gone through TESA without sperm recovery, and the men with a history of previous unsuccessful needle biopsy had SRR 45.8% while the ones with no previous biopsy had SRR of 38,5% (p = 0.456, NS). Sertoli-cell only as the histopathological diagnosis in the previous unsuccessful biopsy led to a SRR of 29% and spermatogenic arrest to 44% (NS). The results are shown in table 7.

The men with preoperative treatment for hypogonadism, mostly aromatase inhibitor and/or hCG, reached SRR of 42.5% and the men without medical treatment 41.5% (NS). In our study, no learning curve was observed, as the SRR of the first 33 men was 39%, the next 34 was 39% and the last 33 was 48% (p=NS).

There was some descriptive data on the pregnancies in study I. Of the 42 men with successful biopsy, 32 had attempted ICSI with the cryopreserved sperm with their female partner. 22 couples had reached at least one live birth, which was consistent with live birth rate of 69%.

The most common complication was infection; four men received oral antibiotics for surgical wound infection, two for epididymitis. There were three hematomas, two of which healed without interventions, and one was explored. The overall rate of surgical complications was 9%. Overall, our sperm recovery results were very good, and the adverse effects were mostly mild.

Table 7. Study I results. SCO= Sertoli-call-only, SA= spermatogenic arrest (adapted from study I)

Etiology	Sperm recovery rate% (n)	Cumulative Live birth rate (n)
Idiopathic, SCO	29% (16/56)	73% (11/15)
Idiopathic, SA	44% (4/9)	50% (2/4)
Klinefelter sdr.	40% (6/15)	50% (1/2)
Y-deletion AZFc	57% (4/7)	33% (1/3)
Cytotoxic and radiation	67% (2/3)	50% (1/2)
Cryptorchidism	90% (9/10)	86% (6/7)
Overall	42% (42/100)	69% (22/33)

5.3 Study II population characteristics

To assess the ICSI treatment results of couples with different forms of male factor infertility, we studied 463 ICSI treatments carried out at Tyks. There was no statistical difference between the mean ages of the men in different study groups. In the OAT group (n = 340), the mean age was 36.2, in the TESA group (n = 52) 35.4 and 31.7 in the micro-TESE group (n = 71). BMI was similar in all the groups, 27.5 kg/m2 in the OAT group, 26.8 kg/m2 in the TESA group and 27.3 kg/m2 in the micro-TESE group. There was a statistical difference between the groups in primary vs. secondary infertility of the male partner; in the micro-TESE groups, 89,5% of the men had primary infertility, while the percentage was 75% in OAT group and 64,4% in TESA group (p = 0.001).

The BMI of the female partner was very similar in the OAT group, in the TESA group and in the micro-TESE group (25.4 kg/m2, 26.4 kg/m2 and 25.1 kg/m2, respectively), with no significant differences. The age of the female partner was slightly higher in the OAT group, 31.8, than in the TESA group, 31.4 and in the micro-TESE group 31.7, p = 0.022. The men in the micro-TESE group had mostly primary infertility (89.5%) whereas in the OAT and TESA groups the percentage (of primary infertility) was significantly lower, 75% and 64.4% respectively (p = 0.001).

There were more patients undergoing antagonist protocol IVF stimulation in the OAT group, 61.9%, and the micro-TESE group 51.1% than in the TESA group, 28,9% (p = 0.0001). The total dose of gonadotropins administered did not show any statistical difference between the three groups (NS). The data are shown in Table 8.

Table 8. Study II population characteristics. (Adapted from study II)

Parametres	OAT	TESA	MicroTESE	p-value
Age of women, mean (SD)	32.8 (4.4)	31.4 (4.7)	31.7 (4.4)	0.022
Age of men, mean (SD)	36.2 (6.8)	35.4 (6.4)	34.3 (6.0)	NS
BMI of women, mean (SD)	25.4 (4.7)	26.4 (4.8)	25.1 (4.6)	NS
BMI of men, mean (SD)	27.5 (4.8)	26.8 (3.8)	27.3 (5.0)	NS
Primary infertility (women)	73.8%	62.2%	82.5%	NS
Primary infertility (men)	75.0%	64.4%	89.5%	0.01
Antagonist protocol	61.9%	28.9%	5.1%	0.0001
Total dose of gonadotropin (mean)	1900 (711)	1844 (602)	1913 (624	NS
Days of stimulation, mean (SD)	9.6 (1,8)	10.0 (1.9)	9.6 (1.3)	NS

5.4 Study II MD-TESE-ICSI treatment outcome

In study II assessing the clinical outcome ICSI severe male infertility, there was no difference seen in the mean number of oocytes collected between the groups, 11.8 in OAT group and TESA group, and 11.9 in micro-TESE group, with mean number of 8.5 (OAT) to 9.1 mature oocytes (TESA and micro-TESE). The fertilization rate was 66.5% in the OAT group, 68.3% in the TESA group, and 62.8% in the micro-TESE group (NS).

The rate of embryo transfer cancellations was significantly higher in micro-TESE group compared to the other groups, 32.4% vs 18.2% in the OAT group and 13.5% in the TESA group (p = 0.02). The most frequent reasons for cancellations were high ovarian stimulation response, planned freeze-all and COVID-19 shutdown.

The groups had similar numbers of embryos transferred per treatment cycle, single embryo transfer rate was 96% in OAT group, 93.3% in TESA group, and 93.5%, in the micro-TESE group. The maximum number of embryos transferred in any cycle was two. In the OAT group, the positive pregnancy test rate was 41.4%, clinical pregnancy rate 33.1% and live birth rate (LBR) was 23.7%. In the TESA group, these numbers were 44.4%, 33.3% and 28.9%, and in the micro-TESE group 39.6%, 27.1% and 25.0%. These small differences did not reach statistical significance. The results are shown in table 9.

No differences were observed between the obstetrical outcomes of the ICSI treatment groups. Except for one twin pregnancy in the OAT group, the other pregnancies in all the study groups were singleton pregnancies. In the OAT group, most pregnancies were full term, with three 33–36-week preterm deliveries. 8 pregnancies lasted more than 42 weeks. In the TESA group, all the pregnancies were full term with no preterm or 42+ gestational week births. In the MD-TESE-group, one pregnancy went overdue, and a single delivery was preterm between gestational weeks 33–36. There were six children with very low birth weight of less than 1500 g in the largest study group (OAT), while the children in the other groups were all more than 2500 g. In the MD-TESE group, one child weighed more than 4000 g, as did 7 children in the OAT group. There were no stillbirths in the study. The obstetric results are shown in table 10.

Overall, we found similar results in all the groups of ICSI treatments studied, regardless of the severity of the male factor infertility.

Table 9. Laboratory results of study II. OPU= ovum pick-up, ET= embryo transfer (adapted from study II)

Parametres	OAT	TESA	MD-TESE	P-value
OPU (n)	**340**	**52**	**71**	
Oocytes (n)	4 018	619	843	
Oocytes mean (n)	**11.8**	**11.9**	**11.9**	
Mature (n)	3 104	442	643	
Mature mean (n)	**9.1**	**8.5**	**9.1**	
Normal fertilized (n)	2 048	302	404	
Fertilization rate (%)	**66.0%**	**68.3%**	**62.8%**	NS
embr. transferred (n)	289	48	51	
No. of frozen emb	1 043	143	239	
Mean no. of viable embryos	**3.9**	**3.7**	**4.1**	
ET (n)	278	45	48	
Cancelled (N)	62	7	23	
Cancelled %	18.2%	13.5 %	32.4%	0,02
Pregnacy tests Positive (n)	115	20	19	
Rate of Pregnancy tests Pos/ET	**41.4%**	**44.4%**	**39.6%**	NS
Clinical Pregnancies (n)	92	15	13	
Rate of Clinical Pregnancies/ET	**33.1%**	**33.3%**	**27.1%**	NS
Live births/Ongoing pregnancy (n)	66	13	12	
Rate of Live births/ET	**23.7%**	**28.9%**	**25.0%**	NS

Table 10. Obstetric outcome of study II. (Adapted from study II)

Parameters	OAT	TESA	MD-TESE	P-value
No. of births	66	13	12	91
Singletons N	65	13	12	NS
Birthweight				
• 1500 g or Less	6	0	0	NS
• 1501–2500 g	0	0	0	NS
• 2501–4000 g	48	13	11	NS
• More 4000 g	7	0	1	NS
	61	13	12	86
Gestational Age				NS
• 22–27 wk	0	0	0	
• 28–32 wk	0	0	0	
• 33–36 wk	3	0	1	
• 37–41 wk	50	13	10	
• 42+ wk	8	0	1	
	61	13	12	

5.5 Study III population characteristics

To assess the use of testicular diffusion weighted imaging in predicting the presence of spermatogenesis, we studied men with positive or negative sperm recovery in MD-TESE and fertile control men. The mean age of the men with positive sperm recovery (SRR+) was 29.5 years (n = 10). The mean age of the men with no sperm recovered (SRR-) was 34.1 years (n = 11). The mean age of the men in the control group was 34.8 (n = 10). Body mass index (BMI) was the same, 28 kg/m2 in all the study groups. The mean age of the men with NOA was 31.5 years. The age differences between the groups were not statistically significant (p = 0.18).

The study men had complete azoospermia in at least two ejaculates. The control group had normospermia with an average total motile sperm count 225 million per ml (variation 78–364million/ml) The etiology of the azoospermia in the study group was KS in 10 men, in 8 men the azoospermia was unexplained, two men had Y deletion of the AZFc region, and one had cryptorchidism operated. The histopathological finding was Sertoli-cell-only in 12 men, spermatogenic arrest in 3 men, hypospermatogenesis in 2 men and Leydig cell hyperplasia in 4 men. Study III population is shown in table 10.

Table 10. Study III population characteristics. SR+= sperm recovery positive, SR-=sperm recovery negative. (Adapted from study III)

	MD-TESE SR+	MD-TESE SR-	Fertile
Age (years)	34.1	29.5	34.8
Body mass index (kg/m2)	27.9	28.1	27.6
Idiopathic NOA (n)	2	6	0
Klinefelter syndrome (n)	5	5	0
Y-del. AZFc (n)	2	0	0
Cryptorchidism history (n)	1	0	0
PAD Sertoli-cell-only (n)	4	8	0
PAD maturation arrest (n)	1	2	0
PAD Leydig cell hyperplasia (n)	2	2	0
PAD hypospermatogenesis (n)	2	0	0
Total motile sperm count (n)	0	0	225 million (78–364 M)

5.6 Study III ADC results

5.6.1 ADC low b-value

The averaged ADC values were compared between healthy controls and NOA men (combined SR+ and SR-patient groups), as shown in Figure 6a. The difference between these groups was statistically significant (P = 0.0041).

The averaged ADC values were also compared between healthy controls, SR+ group, and the SR- group separately as shown in Fig. 6b. The only statistically significant difference was found between healthy controls and the SR- group (P = 0.0040), but not between SR+ and SR- groups (P = 0.35).

The averaged ADC values were also compared between different etiologies KS, NAS, SA, and Y-deletion AZFc, as shown in Figure 6c. There were no statistically significant differences between the groups.

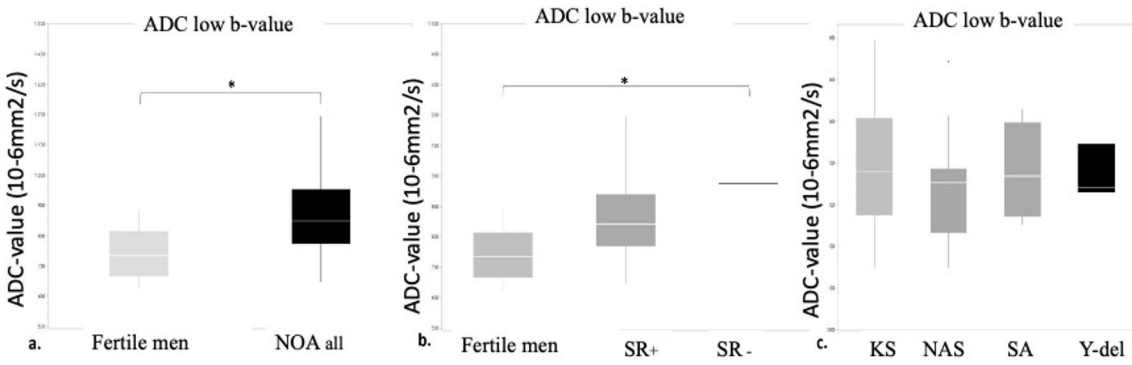

Figure 6a–c. ADC low b-value (10-6 mm2/s). SR+= sperm recovered in micro-TESE, SR- = no sperm recovered in micro-TESE, KS= Klinefelter syndrome, NAS= idiopathic non-obstructive azoospermia, SA= spermatogenic arrest, Y-del = Y chromosome microdeletion AZFc.

5.6.2 ADC medium b-value

The averaged ADC values were compared between healthy controls and NOA men (combined SR+ and SR-patient groups), as shown in Fig. 7a. The difference between these groups was statistically significant ($P < 0.0001$).

The averaged ADC values were also compared between healthy controls, SR+ group, and the SR- group separately, as shown in Fig. 7b. A statistically significant difference was found between healthy controls and the SRR+ group ($P = 0.0003$) as well as between healthy controls and the SRR- group ($P = 0.0001$), but not between the SRR+ and SRR- groups ($P = 0.19$).

The averaged ADC values were also compared between different etiologies KS, NAS, SA, and Y-deletion, as shown in Fig. 7c. There were no statistically significant differences between these groups.

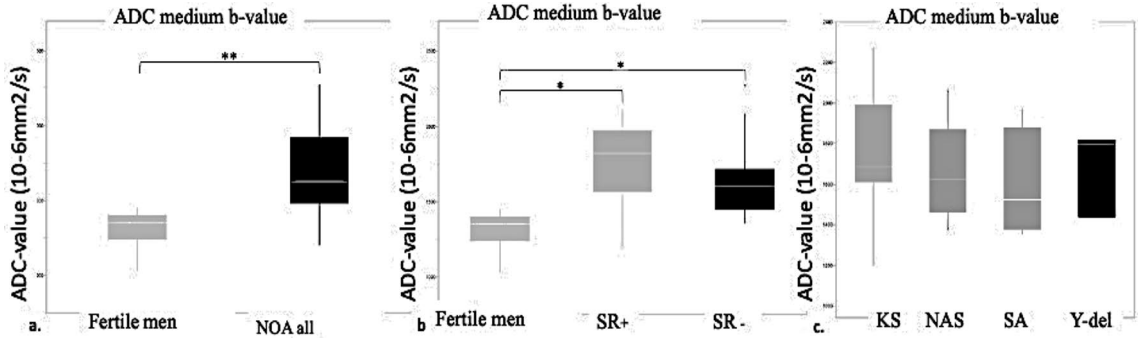

Figure 7a-c. ADC medium b-value (10-6 mm2/s). SR+= sperm recovered in micro-TESE, SR- = no sperm recovered in micro-TESE, KS= Klinefelter syndrome, NAS = idiopathic non-obstructive azoospermia, SA= spermatogenic arrest, Y-del = Y chromosome microdeletion AZFc.

5.6.3 ADC high b-value

The averaged ADC values were compared between fertile controls and NOA men (combined SR+ and SR-patient groups), as shown in Fig 8a. The difference between these groups was statistically significant ($P < 0.0001$).

The averaged ADC values were also compared between fertile controls, the SR+ group, and the SR- group separately, as shown in Fig 8b. A statistically significant difference was found between healthy controls and the SR- group ($P = 0.0003$) as well as between fertile controls and the SR+ group ($P = 0.0001$), but not between the SR+ and SR- groups ($P = 0.16$).

The averaged ADC values were also compared between different aetiologies KS, NAS, SA, and Y-deletion, as shown in Fig 8c. There were no statistically significant differences between these groups.

The ADC values are shown in detail in Table 11. Overall, we found statistical differences in ADC high, median, and low b-values between fertile men and men with NOA. The ADC values were significantly lower in fertile controls than in men with NOA, irrespective of success in micro-TESE. There were no differences between different NOA aetiologies (unexplained Sertoli-cell-only, spermatogenic arrest, Klinefelter syndrome, cryptorchidism, or microdeletion of the Y chromosome) or between the men with successful micro-TESE and the men with no sperm recovered in micro-TESE.

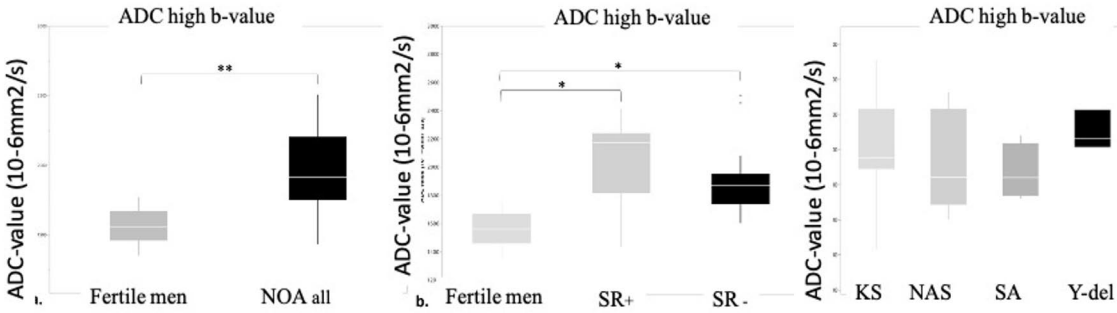

Figure 8a-c. ADC high b-value (10-6 mm2/s). SR+ = sperm recovered in micro-TESE, SR- = no sperm recovered in micro-TESE, KS = Klinefelter syndrome, NAS = idiopathic non-obstructive azoospermia, SA = spermatogenic arrest, Y-del = Y chromosome microdeletion AZFc.

Table 11. Apparent diffusion coefficient (ADC) values of study III. (Adapted from study III)

Mean ADC values	High ADC (B2000)	Median ADC (B1500)	Low ADC (B500)
NOA ALL	1966,82	1689,56	874,15
Fertile controls	1562,79	1313,52	737,98
Unexplained Sertoli-cell-only (6)	1912,69	1616,48	827,15
Klinefelter syndrome (8)	2033,02	1772,03	854,48
Unexplained spermatogenic arrest (2)	1869,87	1568,48	851,55
Y-chromosome microdeletion AZF (2)	2079,24	1856,65	971,02
Sperm recovery +	2043,73	1759,26	851,59
Sperm recovery -	1899,9	1629,86	891,92

6 Discussion

6.1 Sperm recovery rate in MD-TESE

The data from our study I show that as in many other studies described earlier (p. 28–29 (Bernie, Ramasamy, and Schlegel 2013; Achermann, Pereira, and Esteves 2021; H.L. Zhang, Zhao, et al. 2021)), our study subjects' SRR also greatly depends on their NOA aetiology. Our results in unexplained NOA with different histopathological groups were similar to the results in other studies (p. 28–29, (Guler et al. 2016; Aydin et al. 2015; Abdel Raheem et al. 2013)). The patients with the lowest chance of sperm recovery were the men with Sertoli-cell-only and idiopathic NOA (29%). As more than one in four patients will have sperm frozen in the worst group, this prognostic information does not offer significant help in excluding patients from the MD-TESE operation.

In study I, we found that a previous TESA did not predict a worse SRR, and in a half of our patients, MD-TESE was the first attempt of surgical sperm retrieval. The biopsy methods and devices may have varied in the referring clinics. As stated earlier (p. 35), a testicular biopsy is not necessary NOA cases.

The patient age was not a limiting factor to predict MD-TESE success in our study. As discussed on p. 31, in general population of non-Klinefelter men, age does not have an adverse effect on SRR (Ramasamy et al. 2014). We seem to however have higher SRR results in KS patients compared to the average SRR, and to the SRR of Klinefelter patients in some other studies (Corona et al. 2019; Chehrazi et al. 2017; Madureira et al. 2014), which may be due to the fact that we co-operate with the paediatric endocrinologists to reach adolescents with KS and offer them a chance of fertility preservation, when feasible. We have decided not to operate adolescents under the age of 20, since the SRR seems to remain favourable throughout young adult years (p. 31) (Franik et al. 2016; Bryson et al. 2014).

6.2 The role of medical treatment

In study I, we were unable to find difference between the NOA men with medically treated hypogonadism and the ones with normal hormone status. The study design per se is not adequate in investigating the role of medical treatment, as we treated all

the hypogonadal men as suggested by large centres at the time. Our treatment options included aromatase inhibitors, human chorionic gonadotropin and selective oestrogen receptor modulators depending on the treatment response (alleviation of symptoms and serum testosterone level). Aromatase inhibitors were the preferred treatment option due to the affordable price and the peroral administration route. There were occasional issues with mild side effects, i.e., fatigue, loss of libido and sleep disturbances, which rarely led to discontinuation of the medication.

A substantial limitation of our study was the inconsistency of the transmission of information to us due to retrospective nature of the study. We were unable to collect the untreated hormonal status of every participant, and not all follow-up laboratory results were available to us. We also relied on several different laboratories, which may have affected the threshold hormone levels of the study subjects.

Currently the evidence seems insufficient to support the use of medical treatment without individual consideration and counselling (Caroppo and Colpi 2021a). It would require a vast study population of different patient subgroups of NOA men with abnormal hormone status attending MD-TESE to carry out a randomised controlled study investigating the medical treatment options and their safety and efficacy prior to MD-TESE. Considering the current evidence (Shiraishi 2015) (p. 23), a randomised protocol might be considered unethical in many centres.

6.3 Frozen or fresh sperm?

There are several pros and cons to consider when deciding on the use of fresh or frozen sperm from MD-TESE. The fresh sperm is not subject to the risks of freezing, including the potential loss of sperm or the costs and human resources needed for freezing and thawing. The advantages of freezing include time and costs saved if the first operation will not lead to live birth, easy scheduling of the treatment of the female partner and the possibility to ship the frozen samples to other clinics. The surplus sperm should be frozen also in fresh cycles, to avoid reoperations.

In our studies I and II, the outcome of ICSI using frozen-thawed testicular sperm was similar to the previous reports on MD-TESE-ICSI. We have not had any incidents where the frozen sperm have not been found after thawing. However, the studies were not designed to investigate the difference between fresh and frozen sperm and no such results can be extrapolated from our data. The need for sufficient training of the laboratory staff performing MD-TESE-ICSI is evident to ensure excellent results.

The debate on treatment results depending on the use of fresh or frozen sperm seems to be an ongoing debate, with more data mildly favouring the use of fresh sperm (Schlegel et al. 2004; Z. Zhang, Jing, et al. 2021) (p. 26). In our retrospective,

case-controlled study (II), there was a clear bias as the ejaculated sperm was fresh while the testicular sperm was frozen. There was no significant difference favouring the ejaculated, fresh sperm. These results in combination with the clinically mild differences in larger trials have reassured us to continue with our current practice of freezing the MD-TESE samples for future use.

6.4 ICSI outcome after micro-TESE

In study I, we found differences in ICSI outcome in the different subgroups of men but there were insufficient subject numbers per diagnosis to conclude much from these observations. We had poor outcome with Y deletion AZFc men's sperm, which at the time we thought was a coincidence but has later been repeated in other studies, as described on page 27 (H.L. Zhang, Zhao, et al. 2021). In study II, we did not investigate the NOA diagnoses due to the small number of study subjects in each diagnostic group. Data on ICSI results after different histopathological and aetiological subgroups is required to improve patient counselling prior to MD-TESE.

Reporting the ICSI results after MD-TESE are subject to major variation. In study I, we chose to concentrate on the chance of a male attending MD-TESE leading to MD-TESE-ICSI to father a biological child. 22 men of the 100 men participating had a biological child during the follow-up time, which varied greatly from 9 years to 24 months. 22% was higher than 13.5% of the Belgian group (p. 27 (Vloeberghs et al. 2015)), but both analyses have the same problem of drop-out from the treatment. There were also young fertility preservation patients in our study (5/42), making it difficult to make comparisons with pure infertility patient material.

In study I, 69% of the couples treated had at least one live birth. There were one to three MD-TESE-ICSI cycles, including the frozen embryo transfers. More than half had only one ICSI attempt, and some had discontinued the treatment. Male infertility is a condition associated with substantial psychological stress and thus, a risk of drop out. Psychological support and sound information on the treatment results should be given to increase the couples' resilience to tolerate the inevitable time delays and disappointments during the treatment (Wibowo, Johnson, and Wassersug 2016).

The data from our study as well as from other studies have shown that even in a setting of absolute male factor infertility, advancing age of the female partner is associated with worse ICSI outcome. Donor sperm treatment may be associated with higher live birth rate when the female partner is over the age of 40 (Bocca et al. 2017).

In study II, we found the fertilization rate comparable to that of previous reports (Ishikawa, Shiotani, et al. 2009; H.L. Zhang, Zhao, et al. 2021)(discussed p. 29–30). The hypothesis was to find a more marked decrease in fertilization rates in the more

severe male infertility diagnoses. However, there was only a slight tendency towards lower ICSI fertilization in the MD-TESE group, and the pregnancy rate and the live birth rate were similar. The previous studies have shown conflicting results in terms of fertilization and live births (Yalcin et al. 2017; Corona et al. 2019; Almekaty et al. 2019), but in many of them, the setting has been different or the NOA group has included different forms of sperm retrieval. While our patient numbers in the groups with testicular sperm were limited, our strength was the single-unit real-life setting.

In study II, there were more cancelled embryo transfers in the MD-TESE group in comparison to the other groups, which was unexpected and difficult to explain. The higher cancellation rate in the micro-TESE-ICSI group was unexpected. Among the causes of cancellations were high ovarian response, pre-planned freeze-all and COVID-19-related situations (patient-related or due to shut-down policy). The high number of COVID-19 cancellations in the NOA group would have been purely coincidental, but the female factor cancellations are expected, as they were mostly fertile and younger that the female partners in the other groups.

There is a possible selection bias in the NOA group of study II. Since Turku University Hospital is a tertiary centre and was among the first clinics in the Nordic countries to start MD-TESE and still performs the largest number of MD-TESE operations in Finland, majority of the frozen MD-TESE samples are shipped to other Finnish clinics. However, MD-TESE-ICSI treatment cycles with frozen samples with very low sperm yield are often performed in Turku, which may lead to the slightly higher failed fertilization tendency in this group. Again, the male and female partners were oldest in the OAT group, which may compensate for this difference.

6.5 Obstetric outcome

For study I, we received descriptive first-hand information on the pregnancies from the families treated. There were no major complications in the 2nd and 3rd trimester, and the 22 children were born healthy. The twinning rate was however well above the 5% in the Finnish general ART statistics in the same period. We did not have access to the treatment data, but we assume there were more double embryo transfers than in ICSI treatments performed for other, less severe forms of male infertility. In study II, with the data exclusively from Turku University Hospital, there was no difference in the number of embryos transferred per ICSI cycle. With the current evidence showing that MD-TESE-ICSI results are equal to other ICSI results, the safer approach of single embryo transfers should always be primarily offered for MD-TESE-ICSI patients in all clinics.

The obstetric data collected in study II showed that most of the children born after severe male factor infertility treatments reached full term and the birth weight was within the normal range. Conventional TESE compared to TESA has in previous

studies agreed with these results of not finding significant differences in the obstetric outcome of the children born to men with OA or NOA (Tsai et al. 2015).

There were several reasons for choosing a case-control retrospective single-centre approach. Studying a novel method, the single-centre data was less prone to learning curve effect or difficulties with data collection. The complete number of cycles was good, but the number of cycles in TESA and MD-TESE groups was not sufficient to provide significant differences. The numbers were, however, large enough to conclude that clinically the differences, if any, are small and not essential in the patient care. The results of the frozen embryo cycles of these couples still need to be investigated to reach a reliable estimate of cumulative live birth rate per cycle. Also, there is a demand for a well-designed prospective study with a larger sample size to estimate the live birth rates per NOA diagnosis.

Whereas we were not able to see any particular reason for concern in the health of the children born after MD-TESE-ICSI, there are many unanswered questions to be studied among the children born to fathers with NOA. As Y chromosome microdeletion is consistently passed on to male offspring, genetic counselling is needed prior to MD-TESE and the MD-TESE-ICSI treatment. KS is the other well-known genetic cause of NOA where genetic counselling is necessary, but more to inform on the health issues of the man than the next generation, as the children born to fathers with KS seem to have no significant health risks according to the current knowledge (Madureira et al. 2014). With the novel sequencing techniques, a growing number of men with idiopathic NOA may receive a diagnosis potentially passed on to the next generations, leading to increasing need for genetic consultation prior to MD-TESE (Krausz and Chianese 2014).

6.6 Complications of MD-TESE

In study I, we evaluated the complication rate of the MD-TESE operations. The overall infection rate 6% raised concern. The comparisons with other studies are difficult to make since the complications are rarely reported (Achermann, Pereira, and Esteves 2021; Ishikawa, Yamaguchi, et al. 2009). We started administering routine antibiotic prophylaxis of intravenous cefuroxime and metronidazole after 45 MD-TESE operations, leading to a reduced rate in the next 55 consecutive surgeries. The complication rate was collected by searching our patient database, enabling a reporting bias in cases where a minor infection was treated in a local healthcare unit.

MD-TESE, as described earlier, may temporarily decrease serum testosterone level (Ramasamy, Yagan, and Schlegel 2005; Ishikawa, Yamaguchi, et al. 2009). The men participating in study I were advised on the symptoms of low testosterone and the ones presenting with these symptoms were asked to contact us to control the

serum testosterone concentration. The men with previously low serum testosterone level were referred to the laboratory routinely. None of the men with initially normal hormone status developed hypogonadism during our follow up time of 12 months, to our knowledge. It is however possible, that men with NOA with normal baseline hormone status and history of MD-TESE are at an elevated risk of hypogonadism, but more studies are needed on the long-term health of these men.

6.7 Predicting MD-TESE prognosis

The data from these studies are consistent with previous ones showing that male age, BMI, a history of failed needle biopsy, serum FSH, testosterone, or other hormone levels do not reliably predict MD-TESE SRR (p 31–35 (Ramasamy et al. 2014; Iwatsuki et al. 2017; Bernie, Ramasamy, and Schlegel 2013; Ramasamy, Lin, et al. 2009)). Artificial intelligence-based and other multivariable models need to be validated and perhaps commercialized to make their way into the clinical practice of MD-TESE preoperative planning and decision-making (Zeadna et al. 2020; Zheng et al. 2023). The trials combining testicular mapping for tailoring the most effective sperm retrieval method will potentially lead to multiple operations, making them inconvenient and expensive (C.F. Jensen et al. 2016).

In study III, we were aiming to assess the potential of the testicular functional MRI, especially diffusion-weighted imaging (DWI) in predicting the presence of spermatogenesis via possible correlation of ADC and NOA and further assess possible differences in ADC according to NOA aetiology.

While the previous studies assessed the DWI parameters according to histopathological subtypes (Ntorkou et al. 2019), this was the first attempt at differentiating different clinical NOA diagnoses. As the testicular microscopic and macroscopic changes are unique to each of these diagnoses, we were expecting to see the changes in their ADC values. The Klinefelter testis is very small and there is often Leydig cell hyperplasia or hyalinization. In SA, the number of meiotic cells is vast while there are no germ cells in a SCO testis. We expected to see great variation within the measured ADC values, but no significant difference was found.

There were no significant differences between the men with NOA whose MD-TESE was successful compared to the ones without MD-TESE success. The difference between the men with normal fertility and the men with NOA was however significant. Since there was no OA group, the study should be repeated, and an OA group included to recommend the method to distinguish between OA and NOA.

In the Ntorkou study (described p. 33(Ntorkou et al. 2019)), the group was able to show difference between NOA men with and without sperm recovery success in MD-TESE. Their setting was different from ours; they had very small number of

SCO and KS patients and they made the comparisons between testes, not patients. There was in addition great overlap in the ADC values between the testes with and without sperm recovery, making it difficult to adopt the method to clinical practice. In contrast to our setting, they compared histopathological diagnoses whereas we mainly made the comparisons between clinical diagnosis groups.

There are methodological limitations to consider when interpreting the results of study III. As it was a preliminary study, the sample size may have been insufficient to show potential differences between the diagnosis groups. We were also unable to age-match the different study subject groups. As testicular ADC seems to rise with male age, this may form a bias between the groups. Thirdly, not all the measurements were very easy to make, especially in the smallest Klinefelter testicles, giving rise to the question whether all of them were equally reliable.

It is clear, a larger study with sufficient sample size is called for to verify the results. It would ideally include groups of all the major NOA aetiologies and an OA group to clarify whether ADC values are useful independently or as a part of a multivariant model in assist NOA diagnosis and MD-TESE counselling, and decision-making, and whether ADC values are reliable in differentiating between OA and NOA.

We showed in Study III that diffusion ADC-values were able to differentiate non-obstructive azoospermia testes from those with normal spermatogenetic function. In our study, ADC was unable to reliably differentiate the underlying diagnoses of NOA or identify the most potential candidates for MD-TESE with the best chance of sperm recovery.

6.8 Future aspects in MD-TESE

After more than twenty years of MD-TESE research, more than 50% of the men operated will not have sperm retrieved, and only few clinical factors are useful in identifying these men (Punjani, Kang, and Schlegel 2021). An externally validated, reliable prognostic tool to assist in more accurate patient selection is desperately needed.

Artificial intelligence (AI) is a term used to describe many processes where computerized technology is programmed to mimic the functions of human brain. One of the most useful functions is to recognize previous patterns and outcomes and combine data to form prediction models. These models show promise but need to be validated and developed further to be readily available and simple to use in clinical practice.

Image and form recognition quality of AI have not yet matched human skill in fertility laboratory studies. Sperm cell recognition may become a useful tool, but the

sensitivity and specificity have not yet reached sufficient level to be adopted to clinical practice in MD-TESE (Diaz et al. 2022).

Seminal plasma metabolites, RNAs and many other biomarkers have been found to have potential in predicting sperm recovery in testicular biopsies, most importantly MD-TESE (Y. Zhang et al. 2022; J. Li et al. 2023). The results need to be confirmed in multicentre studies and further developed into convenient, reliable, and financially feasible tests to be used in clinical practice.

Next generation sequencing techniques will make idiopathic NOA less frequent, as the chance of identifying underlying genetic causes increases (Kasak and Laan 2021). Genetic screening panels are likely to develop in the next years to become a part of routine assessment prior to MD-TESE. Recognizing different aetiologies of NOA will lead to better patient counselling and prognosis of consequent ICSI treatment results, but also to understand the effect that the NOA diagnosis may have on the offspring. Medical treatment prior to NOA may prove effective to males with certain genotypes, making it possible to aim the treatment more precisely to ones who will benefit from it.

7 Conclusions

1. MD-TESE is an effective method of sperm retrieval for men with non-obstructive azoospermia and should be offered to enable them a chance of biological fatherhood. The MD-TESE results of Turku University Hospital are comparable with those of international good quality centres with larger patient numbers than ours. The complication rate of MD-TESE is low.

2. ICSI is safe and effective using sperm from MD-TESE. No significant differences have been detected between the fertilization rate, live birth rate and pregnancy outcome of MD-TESE-ICSI compared with other ICSI treatments. Larger studies are needed to evaluate the health and fertility of the offspring.

3. There is no non-invasive, reliable method to accurately assess the chance of sperm retrieval before MD-TESE. With MRI, ADC-values can be calculated to differentiate men with non-obstructive azoospermia from those with normal fertility, but not to identify those men with NOA whose sperm retrieval is successful by MD-TESE. More studies are needed to develop an externally validated and reliable prognostic tool to assist in more precise patient selection.

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