EVALUATION OF SPERMATOZOA DNA TESTS FOR AN ASSISTED REPRODUCTIVE TECHNIQUES (ART) PROGRAM: CORRELATION WITH SEMEN PARAMETERS AND ART OUTCOME

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SUMMARY

CHAPTER 1

A review of the application of traditional semen parameters for the investigation and diagnosis of male infertility and the role of predictive values in assisted reproductive techniques (ART) is presented. The importance of sperm morphology, with special emphasis on sperm morphology evaluation, is discussed. Also presented is an overview of the physiology of sperm DNA, the process of spermatogenesis, as well as the contribution of the spermatozoon to the embryo. The different causes of sperm DNA damage and techniques to determine DNA damage in spermatozoa are described. A survey is presented of the correlation of sperm DNA with sperm morphology.

CHAPTER 2

All the materials and methods applicable to this study are described. Sperm morphology assessment and two different sperm DNA tests, the chromomycin A3 (CMA3) staining test and the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) assay, are discussed in detail.

CHAPTER 3

Results obtained in this study are presented. Results include the prevalence of abnormal sperm DNA and association with sperm morphology, specifically in the p-pattern and g-pattern morphology groups. Further results include the correlation of sperm morphology and sperm DNA with fertilization *in vitro*, embryo quality and pregnancy outcome. The percentage CMA3 positive spermatozoa (abnormal DNA) and percentage TUNEL positive spermatozoa (abnormal DNA) had a significant negative association with normal sperm morphology. P-pattern and g-pattern morphology groups differed significantly from each other for both CMA3 and TUNEL. A significant positive association between CMA3 and TUNEL was observed.

No association between the percentage normal sperm morphology, percentage CMA3 positive spermatozoa and percentage TUNEL positive spermatozoa and IUI

pregnancy outcome was observed. A significant negative association between the percentage TUNEL positive spermatozoa and IVF/ICSI pregnancy outcome was established. The percentage CMA3 positive spermatozoa had a significant positive (unexpected) association with IVF/ICSI pregnancy outcome. There was no association between the three variables and IVF/ICSI fertilization rates. A significant positive association between the percentage normal sperm morphology and IVF/ICSI embryo quality was found. There was a significant positive association between the percentage CMA3 positive spermatozoa and IVF/ICSI embryo quality (unexpected). The percentage TUNEL positive spermatozoa and IVF/ICSI embryo quality was negatively associated.

CHAPTER 4

Interpretation of the results and future perspectives are discussed. The CMA3 staining test and TUNEL assay has a limited ability to distinguish between the p-pattern and g-pattern morphology groups. P-pattern spermatozoa are more likely to possess poor chromatin packaging and show increased levels of DNA fragmentation, but some p-pattern patients also may have normal DNA and g-pattern patients abnormal DNA. It is recommended that a sperm DNA test should be implemented routinely in andrology laboratories for the clinical diagnosis of sperm DNA damage in patients.

OPSOMMING

HOOFSTUK 1

'n Samevatting wat handel oor die toepassing van tradisionele semen parameters vir die evaluasie en diagnose van manlike infertiliteit, asook die rol van voorspellingswaardes in kunsmatige voortplantingstegnieke word voorgelê. Die belangrikheid van sperm morfologie, met die klem op sperm morfologie evaluering, word ook bespreek. 'n Oorsig van sperm DNS fisiologie, die proses van spermatogenese, sowel as die sperm se bydrae tot die embrio word hier aangebied. Die verskillende oorsake van sperm DNS skade en die tegnieke om sperm DNS skade vas te stel, asook die die korrelasie tussen sperm DNS en sperm morfologie word ook bespreek.

HOOFSTUK 2

Alle materiale en metodes wat van toepassing is op hierdie studie word beskryf. Sperm morfologie evaluering en twee verskillende sperm DNS toetse, die chromomycin A3 (CMA3) kleuringstoets en die "terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) toets, word ook in meer besonderhede aangebied.

HOOFSTUK 3

Resultate wat verkry is tydens hierdie studie word hier uiteengesit. Resultate behels die voorkomsyfer van abnormale DNS en die assosiasie met sperm morfologie, spesifiek in die p-patroon en g-patroon. Verdere resultate sluit die korrelasie van sperm morfologie en sperm DNS met bevrugting *in vitro*, embriokwaliteit en swangerskap uitkomste in. Die persentasie CMA3 positiewe sperme (abnormale DNS) en persentasie TUNEL positiewe sperme (abnormale DNS) het 'n betekenisvolle negatiewe assosiasie met normale sperm morfologie getoon. P-patroon en g-patroon morfologie groepe het betekenisvol van mekaar verskil vir beide CMA3 en TUNEL. 'n Betekenisvolle positiewe assosiasie is tussen CMA3 en TUNEL waargeneem.

Geen assosiasie is tussen die persentasie normale sperm morfologie, persentasie CMA3 positiewe sperme en persentasie TUNEL positiewe sperme en IUI swangerskap uitkomste waargeneem nie. ‘n Betekenisvolle negatiewe assosiasie is tussen die persentasie TUNEL positiewe sperme en IVB/ICSI swangerskap uitkomste vasgestel. Die persentasie CMA3 positiewe sperme het ‘n betekenisvolle positiewe (onverwags) assosiasie met IVB/ICSI swangeskap uitkomste opgewys. Daar was geen assosiasie tussen die drie veranderlikes en IVB/ICSI bevrugting nie. ‘n Betekenisvolle positiewe assosiasie is tussen die persentasie normale sperm morfologie en IVB/ICSI embryo kwaliteit waargeneem. Daar was ‘n betekenisvolle positiewe assosiasie tussen die persentasie CMA3 positiewe sperme en IVB/ICSI embrio kwaliteit (onverwags). Die persentasie TUNEL positiewe sperme het ‘n negatiewe assosiasie met IVB/ICSI embrio kwaliteit getoon.

HOOFSTUK 4

Interpretasie van die resultate en toekomstige vooruitsigte is bespreek. Die CMA3 kleuringstoets en TUNEL toets het ‘n beperkte vermoë om tussen die p-patroon en g-patroon morfologie groepe te onderskei. P-patroon spermatozoa sal heel waarskynlik oor swakker chromatien verpakking en meer DNS fragmentasie beskik. Sommige p-patroon pasiënte mag egter normale DNS toon, terwyl g-patroon pasiënte abnormale DNS het. Die implementering van ‘n sperm DNS toets in andrologie laboratoriums, vir die kliniese diagnose van sperm DNS skade in pasiënte, word aanbeveel.

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ABBREVIATIONS

AB	Aniline Blue
aCGH	Array Comparative Genomic Hybridization
AO	Acridine Orange
ART	Assisted Reproductive Techniques
CMA3	Chromomycin A3
DFI	DNA Fragmentation Index
FISH	Fluorescent *In-Situ* Hybridization
HA	Hyaluronic Acid
H_2O_2	Hydrogen Peroxide
ICSI	Intracytoplasmic Sperm Injection
IMSI	Intracytoplasmic Morphologically Selected Sperm Injection
IUI	Intra-Uterine Insemination
IVF	In Vitro Fertilization
MACS	Magnetic Activated Cell Sorting
MAR's	Matrix Attached Regions
MSOME	Motile Sperm Organelle Morphology Examination
PICSI	Physiologic Intracytoplasmic Sperm Injection
PS	Phosphatidylserine
PVP	Polyvinylpyrrolidone
ROS	Reactive Oxygen Species
RT	Reverse Transcriptase
SCD	Sperm Chromatin Dispersion
SCGE	Single-Cell Gel Electrophoresis
SCSA	Sperm Chromatin Structure Assay
TP's	Transition Proteins
TUNEL	Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labelling
WHO	World Health Organization

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CHAPTER 1

BACKGROUND INFORMATION AND LITERATURE REVIEW

1.1 Male Infertility and Standard Semen Parameters

Infertility is a health concern affecting nearly one in six couples of childbearing age and approximately 10% of the global population. Male-factor infertility accounts for 50% of all cases (Simon *et al.,* 2010; Cassuto *et al.,* 2012; Ribas-Maynou *et al.,* 2012). Regardless of this high incidence, our current knowledge of the basic pathophysiology of male infertility is fairly minimal (Aitken and Bennetts, 2007). The key responsibility of ART (Assisted Reproductive Techniques) is the management of infertility – including male infertility. The primary identification of male-factor infertility is largely based on the assessment of specific sperm characteristics. ART outcome is to a great extent affected by spermatozoa function and quality (Kazerooni *et al*., 2009; Qiu *et al.,* 2012).

Consequently, standard semen parameters, as described by the World Health Organization (WHO, 2010), have been implemented for routine analysis and include sperm count, motility and morphology. For many years the traditional semen analysis was considered as the gold standard for the investigation and diagnosis of male infertility. Although necessary information obtained from conventional semen parameters indicates some degree of the sperm quality, the relationship between these sperm parameters is merely modest predictors of ART outcome, especially because of its high intra-individual variation (Varghese *et al*., 2009; Simon *et al.,* 2010; Oleszczuk *et al.,* 2011).

The main purpose of predictive values is to give infertility patients a realistic understanding of their fertility potential (Coetzee and Kruger, 2007). Today, the classic semen analysis is recognized to be of limited value in describing a couple's fertility status (Simon *et al.,* 2010). A fundamental need is for reliable sperm biomarkers to be developed to determine sperm quality and possibly reveal the origin of some unexplained repeated *in vitro* fertilization (IVF) failures (Lazaros *et al.,* 2011). A clinical biomarker for male infertility may also be helpful when a decision for the most appropriate assisted reproductive procedure has to be made.

1.2 Clinical Importance of Sperm Functional Tests

As previously mentioned, the basic semen analysis (sperm count, motility and morphology) is traditionally applied as the initial step to evaluate male-factor infertility. Male-factor infertility may be the result of structural or biochemical sperm changes. Modifications of the sperm membrane, nuclear abnormalities, cytoplasmic defects, and flagellar disturbances are strongly related to sperm function. The role of spermatozoa as functional gametes depends on many factors. The majority of infertile men may produce spermatozoa, yet these gametes are characterized by functional defects (Aitken and Bennetts, 2007).

Therefore, sperm functional tests have been developed to assess the functional capacity of spermatozoa *in vitro*. These sperm functions include: the acrosome reaction, sperm capacitation, zona pellucida binding, oolemma binding, decondensation, and pronuclear formation (Oehninger *et al.,* 2007). Sperm functional tests provide important information on which clinicians can base their initial diagnosis (Lewis *et al*., 2008).

1.3 Sperm Morphology and Tygerberg Strict Criteria

Sperm morphology is an important semen parameter of male fertility assessment and was correlated to natural *in vivo* fertilization*, in vitro* fertilization, as well as pregnancy outcome (Kruger *et al.,* 1986; Kruger *et al.,* 1996; Menkveld and Kruger, 1996; Coetzee *et al.,* 1998; Avendaño *et al.,* 2009). Sperm morphology evaluation has improved immensely over the past years. Several staining methods are available to assess sperm morphology. The Papanicolaou staining method is recommended, but rapid staining methods, such as the Diff-Quik (Hemacolor®) can also be used. The Tygerberg Strict Criteria method was developed by Menkveld and colleagues for the morphological classification of human spermatozoa (Menkveld, 1987; Menkveld *et al.,* 1990; WHO, 2010).

According to Tygerberg Strict Criteria, the measurements of a morphologically normal spermatozoon during Papanicolaou staining include a smooth oval head, which contains the paternal DNA, with a length of about 3.0 - 5.0 µm and a width of approximately 2.0 - 3.0 µm. The sperm head contains a well-defined acrosomal region which comprises 40-70% of the anterior head area. The acrosome is characterized by the Golgi complex and hydrolytic enzymes, such as hyaluronidase

and proacrosin. These acrosomal enzymes play an important role during fertilization. The midpiece should be approximately 1.0 µm thick with a length of about 6.0 - 10.0 µm. The mitochondria are located in the midpiece. The straight sperm tail should be about 45.0 - 50.0 µm long (Kruger *et al.,* 1988; Menkveld *et al.,* 1990; Kruger *et al.,* 1996; WHO, 2010). Slight enlargement of the sperm head may occur during Diff-Quik staining. The length of the sperm head is approximately 4.0 – 5.5 µm and the width is about 2.5 – 3.5 µm (WHO, 2010).

Abnormal sperm morphology include: (i) acrosomal defects; (ii) amorphous, pyriform, tapered, dumb-bell, round or double head shapes; (iii) vacuolated heads; (iv) bent or broken necks and midpieces; (v) the presence of cytoplasmic droplets around the head, midpiece or tail, and (vi) bent, coiled or multiple tails (Menkveld and Coetzee, 1995; Menéndez and Marina, 1999; Raja and Franken, 2006).

1.4 Sperm Morphology Groups

The predictive value of sperm morphology, using WHO guidelines or Tygerberg Strict Criteria is well established. Kruger *et al.* (1986, 1988) described three prognostic categories with Strict Criteria which include the poor-prognosis or p-pattern group (1-4% morphologically normal spermatozoa), good-prognosis or g-pattern group (5-14% morphologically normal spermatozoa) and normal-prognosis or n-pattern group (≥ 15% morphologically normal spermatozoa).

Previous studies using the 5% and 15% sperm morphology thresholds (Strict Criteria) showed positive predictive values for IVF success rates. A significant decrease in pregnancy rates was also observed in the p-pattern morphology group (Coetzee *et al.,* 1998; Van Waart *et al.,* 2001; Van der Merwe *et al.,* 2005). Kazerooni *et al*. (2009) demonstrated that normal sperm morphology can be used as an indication of normal sperm function, while increased levels of abnormal spermatozoa in the ejaculate are associated with lower fertilization rates, poor embryo quality and delayed embryo development.

1.5 The Physiology of Sperm DNA

An important function of the human spermatozoon is to successfully transport and deliver the paternal chromosomes to the oocyte (Sousa *et al.,* 2009). The nucleus, within the highly specialized sperm head, contains a haploid set of chromosomes.

The acrosome covers the anterior area of the sperm head. The nucleus comprises almost 65% of the sperm head. Sperm chromatin, within the nucleus, is very tightly compacted due to the unique association among the DNA, the nuclear matrix, and the sperm nuclear proteins. The particular chromatin structure is fundamental to its compaction and stabilization. The greater than ten-fold compaction of sperm chromatin is achieved during the final post-meiotic phases of spermatogenesis (Miller *et al.,* 2010). The DNA molecules are made up of many nucleotides and are involved in carrying the paternal genetic information, which includes the sex-determining X or Y chromosome (Hoogendijk and Henkel*,* 2007).

The structural organization of sperm DNA can be divided into four phases:
Phase 1: Two DNA strands, that shape the chromosomes, are connected to the sperm nuclear annulus, in a process generally identified as chromosomal anchoring. The ring-like nuclear annulus is found only in the sperm nuclei. Chromosomes are arranged in specific DNA sequences within the nuclear annulus. The shape of the sperm nucleus may therefore be influenced by the nuclear annulus (Hoogendijk and Henkel*,* 2007).

Phase 2: Fractions of the nuclear matrix and protein structural fibres are attached to the DNA by matrix attachment regions (MAR's) and chromosomes are arranged into DNA loops. The DNA within these loop domains is organized into densely packed toroids. It has been suggested that DNA loop domains are essential for sperm DNA functioning. However, this suggestion is still not clear. DNA loop domains may have a regulatory function during transcription and DNA replication in early embryonic development (Hoogendijk and Henkel*,* 2007).

Phase 3: The DNA loops are condensed into tightly packed chromatin structures, because of protamine binding. DNA-protamine binding is responsible for the formation of toroidal or doughnut-like configurations in which the DNA is highly concentrated. Protamine binding will result in condensation of DNA loop domains in round spermatids (Hoogendijk and Henkel*,* 2007).

Phase 4: Sperm chromatin packaging involves the arrangement of chromosomes in the mature sperm nucleus. In human spermatozoa the unique DNA sequences are to be found at the nuclear base where centromeres are positioned centrally and telomeres are located peripherally. Chromosome alignment varies among different species (Hoogendijk and Henkel*,* 2007).

1.6 Spermatogenesis

Spermatozoa are produced during the process of spermatogenesis, in which the genome of these gametes condenses from being diploid to haploid cells. Morphological and functional modifications also occur. The three major events during spermatogenesis include proliferation and differentiation of diploid spermatogonial cells (proliferative phase), meiosis, and spermiogenesis (Jégou *et al.,* 2002). Sperm maturation is essential for optimal sperm function (Hoogendijk and Henkel*,* 2007). Damage to the genome of these gametes can happen during any of the developmental events.

1.6.1 Proliferation and Differentiation of Diploid Spermatogonial Cells

Spermatogonial type A cells proliferate into primitive germ cells, known as spermatogonia. These germ cells can be distinguished based on their morphological appearance, which include type A long (A_{long}), type A dark (A_{dark}), type A pale (A_{pale}), type B, primary spermatocytes, secondary spermatocytes, and spermatids. Differentiated spermatogonia cell types consist of A_0, A_1, A_2, A_3, A_4, as well as type B spermatogonia. It has been proposed that type A_0 divide slowly and will remain at the basement membrane to replenish the germ cell line if damage to the testis occurred (Menéndez and Marina, 1999). Consequently, types A_1-A_4 are renewing spermatogonia cells responsible for fertility preservation. Type A will differentiate into intermediate type B spermatogonia. Type B spermatogonia contain much more chromatin than type A spermatogonia. Type B spermatogonia are direct precursors of primary spermatocytes (Menéndez and Marina, 1999; Jégou *et al.,* 2002; Hoogendijk and Henkel*,* 2007).

1.6.2 Meiosis

The process initiates as soon as type B spermatogonia form primary spermatocytes. During this meiotic phase these spermatocytes will undergo two sequential cell divisions, which include meiosis I and meiosis II. During meiosis I each primary spermatocyte will form two haploid secondary spermatocytes. Although these secondary spermatocytes contain only half the chromosome number, its total DNA content is equal to that of diploid cells, since each chromosome contains a pair of daughter chromatids. Thereafter, meiosis II occurs and the chromatids of each chromosome separate into two round spermatids. These spermatids carry a haploid

chromosome number and half the DNA amount of secondary spermatocytes (Menéndez and Marina, 1999). Although regulatory mechanisms are present during meiotic divisions, anomalies in chromosome pairing may contribute to male infertility (Jégou *et al.,* 2002).

1.6.3 Spermiogenesis

During spermiogenesis spermatids differentiate and change morphologically into spermatozoa. This process of spermatid maturation follows once meiosis is completed. The three most important events of this metamorphic phase involve elongation and condensation of the nucleus, development of the acrosome, and the organization of a keratin scaffold enclosing the axoneme and tail (Menéndez and Marina, 1999).

Eighty-five present of DNA-binding proteins, specifically histones, are replaced by basic transition proteins, which in turn are substituted by cysteine-rich protamines (Kazerooni *et al*., 2009). Transition proteins, TP-1 and TP-2, are shown to have an important role in sperm chromatin condensation. The DNA-binding properties of TP's and protamines also contribute to the repair of DNA strand breaks. Round haploid spermatids will therefore ultimately differentiate into highly species-specific spermatozoa. The haploid spermatid exhibits a typical nucleosomal chromatin pattern during the initial stages of spermiogenesis. As the process of spermiogenesis progresses, the beaded chromatin configuration is substituted by smooth chromatin fibres. Condensation of the haploid spermatid genome is a very important step during the final stages of spermiogenesis (Laberge *et al*., 2004).

1.7 Formation of the Male Pronucleus

The male pronucleus is associated with the centrosome. The centrosome is a crucial component of the fertilizing spermatozoon, contributing to the assembly of microtubule within the penetrated oocyte. Another responsibility of the centrosome is the formation of the mitotic spindles during the initial fertilization phase. Only once the paternal genomes unite (syngamy) and migration of the female pronucleus to the male pronucleus on microtubules occurred, fertilization is complete. The centrosome in turn nucleates microtubules to form the sperm aster. The centrosome, as well as the male pronucleus is driven by the growing sperm aster from the cell cortex towards the centre of the oocyte (Barroso and Oehninger, 2007).

1.8 Contribution of the Male Gamete to the Embryo

The paternal genome is transferred to the oocyte in a balanced physical and chemical condition to complement genetic division during embryo development. Although the paternal genome is not effective in the human embryo until day 3 (four- to eight-cell stage), it may influence the embryo at various levels including nuclear, cytoskeletal, and at an organelle level (Raja and Franken, 2006, Thomson *et al.,* 2011). The DNA integrity of human spermatozoa contributes significantly to embryonic growth and fetal health (Kumar *et al.,* 2012).

Good-quality oocytes have the ability to repair nuclear damage in the male gamete. The cytoplasmic and genomic qualities of the oocyte are however influenced by advanced maternal age. The human embryo is well equipped with natural protection mechanisms and pathways which can prevent further embryonic development, if the DNA damage is too severe (Sakkas and Alvarez, 2010). These mechanisms include instant reversal of DNA damage, single-strand lesion repair, base deletion adjustment, nucleotide removal repair, and mismatch correction (Kumar *et al.,* 2012).

Nevertheless, some lesions may possibly be repaired incorrectly or else remain impaired (Meseguer *et al.,* 2011). Fertilization failure or poor embryo quality may occur if decondensation of the sperm DNA, after entering the ooplasm, was unsuccessful (Shafik *et al*., 2006). If critical genes are damaged, it may result in non-viable embryos or early pregnancy loss (Avendaño *et al.,* 2010; Balasuriya *et al.,* 2011).

1.9 Research on the DNA Integrity of Spermatozoa

ART procedures, such as the intracytoplasmic sperm injection (ICSI) technique, offer treatment for infertile couples with severe male-factor infertility (Lewis *et al.,* 2008). ICSI is mainly based on the selection of a motile spermatozoon with a good morphology, using high-power magnification (Avendaño *et al.,* 2010). This technique allows us to side-step the natural selection barrier for genetic, as well as functional sperm defects (Simon *et al.,* 2010). As a result, unknown abnormalities at the sperm DNA level may be ignored and there could be the risk of transferring a genetically abnormal male genome into the oocyte (Avendaño *et al.,* 2009; Speyer *et al.,* 2010).

Recently, screening techniques for the detection of sperm DNA defects have come into view (Varghese *et al*., 2009). The DNA integrity of spermatozoa has been shown to be more predictive at various fertility checkpoints and has a significant effect on the reproductive potential of men, as well as ART success rates (Agarwal *et al.,* 2004; Varghese *et al*., 2009; Meseguer *et al.,* 2011). It has been shown that men with normal semen parameters have lower levels of DNA abnormalities when compared to patients undergoing fertility treatment (Irvine *et al*., 2000). Thus, attempts have been conducted to classify the DNA integrity of human spermatozoa as an important semen parameter for the evaluation and sequential diagnosis of male infertility.

1.10 Causes of Sperm DNA Damage

Many theories have been postulated to explain the origin of DNA damage in human spermatozoa. The DNA of spermatozoa can be damaged during any of its developmental, transport or storage stages (Fernández *et al.,* 2008; Sakkas and Alvarez, 2010). Spermatozoa with damaged DNA can be identified within the testes, epididymis, and the ejaculate. The DNA damage is normally lowest in the testes and increases in the caudal epididymis and ejaculate (Lewis *et al.,* 2008; Sakkas and Alvarez, 2010).

Sperm DNA damage may be triggered by intrinsic, as well as external factors. On an intrinsic level, abnormal genomic material may be the result of DNA compaction or nuclear maturity defects, DNA strand breaks, DNA integrity anomalies, or sperm chromosomal aneuploidies (Shafik *et al*., 2006). The amount of spermatozoa with damaged DNA is predominantly higher in the ejaculate of men with poor quality semen (Varghese *et al*., 2009). As a result, anomalies within the sperm DNA may be associated with abnormal semen parameters (Shafik *et al*., 2006).

1.10.1 Incomplete Chromatin Packaging during Spermiogenesis and Sperm Maturation

A number of nuclear events associated with spermiogenesis may be potential causes of genetic instability in mature spermatozoa (Leduc *et al*., 2008). Small arginine-rich nuclear proteins, known as protamines, are synthesized in late-stage mammalian spermatids (Balhorn, 2007; García-Peiró *et al.,* 2011). Two nucleoproteins, protamine 1 (P1) and protamine 2 (P2), are found in human spermatozoa in more or less equal quantities. An altered P1/P2 ratio or the absence of P2 may be expressed

in infertile men. Spermatozoa displaying an altered P1/P2 ratio are normally more susceptible to stressors and have been correlated with sperm DNA fragmentation (Erenpreiss *et al.,* 2006; García-Peiró *et al.,* 2011). Protamines are primarily identified for their involvement in DNA integrity and compaction of the sperm head (Kierszenbaum, 2001; Leduc *et al*., 2008). During sperm maturation, histones are substituted by protamines and stabilized by intra-inter-molecular disulphide cross-links among cysteine-residues (Nasr-Esfahani *et al.,* 2005).

Sperm chromatin abnormalities can occur at various levels, including histone-protamine replacement, absent protamines, epididymal maturation, and chromatin stability during ejaculation (Kazerooni *et al*., 2009). The modification or absence of chromatin proteins, especially histones, not only lead to anomalies in the chromatin packaging, but also have an influence on sperm quality and fertilization potential.

1.10.2 Impact of Excessive Heat on Sperm DNA Quality

Spermatozoa with improper chromatin packaging are more sensitive and become single-stranded when exposed to stressors, such as extreme temperatures or frequent pH changes. If the sperm DNA is strongly associated with disulphide-rich protamines, it is mostly resilient against denaturation (Varghese *et al*., 2009). Also, damaged DNA denatures more rapidly than intact DNA (Chohan *et al.,* 2004; Fuse *et al.,* 2006).

1.10.3 Influence of Oxidative Stress and ROS on Sperm DNA

Oxidative stress, caused by the production of reactive oxygen species (ROS), seems to have a critical influence on male reproduction. ROS include a variety of metabolic derivatives from the oxygen molecule, including strong oxidants and free radicals (Aitken and Bennetts, 2007). ROS, particularly hydrogen peroxide (H_2O_2), are produced by spermatozoa and seminal leukocytes. Their effect depend on the amount of ROS generated (Shafik *et al.*, 2006; Luconi *et al.,* 2007; Thomson *et al.,* 2011). High levels of semen leukocyte-derived ROS are frequently associated with the retention of residual cytoplasm around the sperm midpiece, as well as alterations of the sperm membrane (Leduc *et al*., 2008).

ROS can influence sperm DNA by inducing double- and single-stranded DNA breaks and thereby cause nucleotide modifications (Thomson *et al.,* 2011). The presence of

double- or single-stranded DNA breaks in mature spermatozoa has been strongly linked to male infertility (Laberge *et al*., 2004). Sperm DNA damage due to oxidative stress can have an influence on both embryo quality and clinical pregnancy rates after IVF and ICSI (Thomson *et al.,* 2011).

1.10.4 Apoptotic DNA Degradation in Spermatozoa

Apoptosis, also identified as 'programmed cell death', is induced by highly specialized Sertoli cells within the seminiferous tubules and influence 50-60% of human germ cells that go into meiosis I. These cells are assigned with the *Fas* type apoptotic markers and should undergo phagocytosis after which they are removed by the Sertoli cells (Sakkas and Alvarez, 2010). Although these germ cells are controlled by the Sertoli cells, they can experience negative feedback activities on them. These damaged germ cells may enter spermiogenesis and eventually end up in the ejaculate (Jégou *et al.,* 2002). Therefore, apoptosis has also been associated with sperm chromatin condensation and DNA fragmentation.

Apoptotic DNA degradation in spermatozoa may be induced during *in vivo,* as well as *in vitro* conditions. *In vivo* apoptosis may occur at various levels, more specifically at testicular, epididymal or seminal level. Hormonal depletion, irradiation, toxic agents, chemicals, heat exposure and elevated testicular temperature are factors responsible for apoptosis on testicular level (Barroso and Oehninger, 2007). The presence of leukocytes and the production of free radicals during sperm migration, inflammation, or infection have been shown to induce apoptosis on epididymal level (Bronet *et al.,* 2012). ROS and antioxidant depletion are causes of apoptosis on seminal level (Barroso and Oehninger, 2007).

1.10.5 Sperm Chromosomal Aneuploidies

The extreme shortening of highly conserved guanine-rich hexameric repeats, known as telomeres, may be a trigger of apoptotic DNA fragmentation (Kumar *et al.,* 2012). Telomeres are located at the end of the chromosome and protect the chromosome from random rearrangements and discourage the recognition of chromosomal ends as DNA breaks. A special type of reverse transcriptase (RT), containing the subunit (Tert) and a RNA script (Terc), is pivotal for the synthesis of telomeric repeats.

A modification of this RT due to genetic deletions or mutations, in conjunction with the loss of Tert or Terc, induces variation of the telomeric end-sequences of newly synthesised chromosomes. This leads to the shortening of telomeric regions of new chromosomes and end-to-end fusion. The result of such a phenomenon also contributes to cell arrest and cell apoptosis (Rodriguez *et al.,* 2005; Shafik *et al*., 2006; Balasuriya *et al.,* 2011).

1.10.6 Influence of External Factors on Sperm DNA Integrity

Lifestyle factors, such as diet choice, excessive alcohol consumption, smoking, caffeine intake, antibiotics, hyperthermia, and air pollution may affect the DNA integrity of spermatozoa (Leduc *et al*., 2008). Similarly, cancer, in addition to other diseases, genital tract inflammation, semen infections, hormonal disorders, and aging are increasingly being linked to sperm DNA abnormalities (Shafik *et al*., 2006; American Society for Reproductive Medicine, 2008; Sakkas and Alvarez, 2010).

1.10.7 Effect of Cryopreservation on Sperm DNA

Sperm DNA fragmentation may occur during the handling and preparation of semen samples for assisted reproduction (Meseguer *et al.,* 2011). Even the presence of some ions in sperm culture media may be associated with DNA fragmentation (Barroso and Oehninger, 2007). Furthermore, cryopreservation of spermatozoa has been widely implemented in assisted reproduction. Chemical and physical changes, such as ice crystal formation, cellular dehydration, and osmolarity fluctuations may be detrimental to the spermatozoa and their DNA (Meseguer *et al.,* 2011).

Chohan *et al.* (2004) confirmed the negative impact of cryopreservation on the chromatin integrity of human spermatozoa. Meseguer *et al.* (2011) also reported on the effect of cryopreservation on sperm DNA and other sperm structures, such as the membrane and mitochondria. Many studies showed that frozen-thawed semen samples have higher levels of sperm DNA damage compared to fresh semen samples. It is also believed that some patients are more susceptible to DNA damage after freezing and thawing procedures than others (Chohan *et al.,* 2004).

1.11 Effect of DNA Damage on Fertilization, Embryo Quality and Pregnancy Outcome

The selection of a spermatozoon with nucleotide or DNA damage during ART procedures may influence the genetic quality of the embryo (Sakkas and Alvarez, 2010). These genetic modifications contribute to impaired implantation and poor embryogenesis (Shafik *et al*., 2006; Lazaros *et al.,* 2011). This may have an impact on a couple's fertility potential and ART outcome (Sakkas *et al.,* 1995; Bianchi *et al.,* 1996; Shafik *et al*., 2006). Sperm DNA damage has also been associated with a high abortion incidence and disease in offspring, such as childhood cancers and autism (Lewis *et al.,* 2008; Thomson *et al.,* 2011).

In Evenson's study it was concluded that when 30% or more of spermatozoa displayed abnormal DNA, the female had difficulties achieving a healthy pregnancy (Evenson *et al*., 1999). According to the sperm chromatin structure assay (SCSA) threshold value for fertility, a DNA fragmentation index (DFI) higher than 30% is reported statistically significant and is associated with poor fertilization rates in intra-uterine insemination (IUI), IVF and ICSI (Fernández *et al.,* 2003; Apedaile *et al*., 2004; Fernández *et al.,* 2005; Oleszczuk *et al.,* 2011). The chance of pregnancy for patients who underwent IUI was significantly increased when the DFI was less than or equal to 27% (Bungum et al., 2004). Previous results also emphasized that IVF or ICSI should rather be considered for patients with a DFI higher than 30%, since the cleavage rate of embryos was significantly lower after insemination (Virant-Klun *et al*., 2002; Evenson and Wixon, 2006). In a study conducted by Kumar *et al.* (2012) it was concluded that couples with a DFI of 26% were able to fall pregnant, but could not sustain the pregnancy which resulted in recurrent pregnancy loss. Bronet *et al. (*2012) also confirmed a correlation between high DFI values and embryo aneuploidies.

According to literature, pregnancy loss also occurs with an increase in the degree of sperm DNA fragmentation as detected by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) assay. This could be the cause of higher miscarriage rates and lower clinical pregnancy rates in infertility patients (Carrell *et al*., 2003). The pregnancy outcome for patients who underwent IVF was significantly lower when TUNEL-positive sperm (36.5%) were used (Henkel *et al*., 2004).

Sakkas *et al.* (1998) found that patients with poor chromatin packaging [> 30% chromomycin A3 (CMA3) fluorescence], had more than double the number of unfertilized oocytes. Previous studies have also shown that the number of CMA3 positive spermatozoa is significantly higher in patients with spontaneous recurrent abortions (Kazerooni *et al*., 2009).

Virant-Klun *et al.* (2002) reported that fertility patients with failed IVF cycles had 65% spermatozoa with single-stranded DNA, as identified by the acridine orange (AO) staining test. Although a negative correlation between spermatozoa with single-stranded DNA and embryo quality exists, the negative effect of single-stranded DNA on embryo quality is not very clear (Virant-Klun *et al*., 2002). However, Lazaros *et al.* (2011) concluded that an increased number of DNA strand breaks are associated with poor embryo morphology at the early cleavage stage. The development of these embryos does not continue further than the six- to eight-cell stage, alongside the incomplete activation of their embryonic genomes. Likewise, Ribas-Maynou *et al.* (2012) demonstrated that double-stranded DNA breaks contribute to chromosomal instability in embryos.

1.12 Techniques to Determine Sperm DNA Damage

Large clinical trials and extensive research are essential for the successful implementation of sperm DNA tests into routine practice. The infinite variability of sperm DNA tests causes a number of challenges. An abundance of various methods, measuring sperm DNA integrity, raises doubt regarding the lack of standardized protocols which may contribute to inter-laboratory variations (Muratori *et al.,* 2010). Also, these results do not reveal the underlying physiological mechanisms of DNA damage in human spermatozoa. Although it is controversial, a universal agreement on the ultimate technique for the accurate evaluation of human sperm DNA integrity has not yet been reached (Chohan *et al.,* 2004; Shafik *et al*., 2006; Muratori *et al.,* 2010).

1.12.1 Techniques to Determine DNA Fragmentation in Spermatozoa

Direct DNA damage in spermatozoa may be the result of double- or single-stranded DNA breaks. Several assays have been developed for the assessment of DNA strand breaks, such as the single-cell gel electrophoresis (comet) assay, the sperm chromatin dispersion (SCD) test, the terminal deoxynucleotidyl transferase

(TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) assay, the acridine orange (AO) staining test, and the sperm chromatin structure assay (SCSA) (Henkel, 2007; Varghese *et al*., 2009; Muratori *et al.*, 2010).

- **Single-Cell Gel Electrophoresis (Comet) Assay**

The Comet assay measures damaged DNA strands and allows the distinction between single- and double-stranded DNA breaks in spermatozoa, depending on whether alkaline (pH > 13) or neutral conditions are applied (Ribas-Maynou *et al.,* 2012). During electrophoresis, the damaged DNA strands separate from intact DNA in the sperm head and expand out of the nucleus into an agarose gel. Intact DNA will remain in the sperm nucleus. The migration of the DNA strand breaks in the agarose depends on the intensity of the DNA damage, thus influencing the comet tail size. Intact DNA is visualized in the comet head and the comet tail contains the damaged DNA (Steele *et al.,* 1999). Evaluation involves the length of the comet tail, as well as the amount of DNA in the tail. A DNA-specific fluorescent stain is applied for visualization (Henkel, 2007).

- **Sperm Chromatin Dispersion (SCD) Test**

The SCD test originated upon the different reactions presented by the nuclei of spermatozoa with fragmented DNA compared to those with intact DNA. When the sperm DNA is undamaged, structured DNA denaturation and removal of nuclear proteins will result in the expansion of the DNA loops in partly deproteinized nucleoids to form large chromatin dispersion halos. The nuclei of spermatozoa with elevated DNA fragmentation fail to display a dispersion halo (Fernández *et al.,* 2005; Parmegiani *et al.,* 2010). Fluorescence microscopy was initially applied in the SCD test to evaluate DNA fragmentation in spermatozoa, when a DNA-specific fluorochrome has been used. This method was revised to use bright-field microscopy after Diff-Quik staining (Fernández *et al.,* 2005; Zhang *et al.,* 2010). Recently, the Halosperm® kit has been introduced as a novel and standardized version of the SCD test. The Halosperm® kit demonstrates improvement in the chromatin staining quality and protection of the tails (Fernández *et al.,* 2005; De la Calle *et al.,* 2008).

- **Terminal Deoxynucleotidyl Transferase (TdT)-Mediated Deoxyuridine Triphosphate (dUTP) Nick-End Labelling (TUNEL) Assay**

The TUNEL assay is based on the adding of labelled dUTP-nucleotides to single- and double-stranded DNA breaks, using the TdT-enzyme. The dUTP-nucleotides are DNA precursors and are incorporated at the 3' –OH ends of DNA strands (Henkel, 2007). Labelled DNA breaks can therefore be identified and reveal DNA fragmentation in spermatozoa by means of fluorescence microscopy or flow cytometry (Muratori *et al.,* 2010).

- **Acridine Orange (AO) Staining Test**

The AO staining test reveals the susceptibility of sperm DNA to denaturation and thereby measures the stability of the sperm chromatin (Virant-Klun *et al.*, 2002; Kazerooni *et al.*, 2009). AO is applied for its metachromatic properties. In the presence of normal double-stranded DNA the separation of AO molecules occur. In this monomeric form of the dye the AO molecules emit green fluorescence. AO molecules bind electrostatically to single-stranded DNA or RNA, forming aggregates. A concentration dependant loss of absorbed energy takes place, causing a metachromatic shift to red-orange fluorescence (Apedaile *et al*., 2004).

- **Sperm Chromatin Structure Assay (SCSA)**

The SCSA also measures the susceptibility of sperm DNA to denature when exposed to certain conditions. This assay is based on partial acid-induced denaturation and staining with AO. Flow cytometry is utilized for the detection and analysis of the AO fluorescence (Simon *et al.,* 2010). An acid-detergent, such as Triton X-100, permeabilizes the sperm membrane, allowing greater AO access to the DNA. AO binds DNA which fluoresces green with double-stranded (intact) DNA and red-orange with single-stranded (fragmented) DNA. The DNA fragmentation index (DFI) describes the percentage of abnormal spermatozoa (red-orange stained spermatozoa) (Fernández *et al.,* 2003; Apedaile *et al*., 2004; Fernández *et al.,* 2005; Oleszczuk *et al.,* 2011).

Although the TUNEL assay and the SCSA can be applied for DNA fragmentation assessment, it is important to emphasize that the TUNEL assay measures existing

DNA damage, whereas the SCSA measures potential DNA damage in spermatozoa. For example, the TUNEL assay reveals the actual amount of DNA strand breaks and the SCSA discloses the vulnerability of DNA to denaturation at sites of DNA breaks (Muratori *et al.,* 2010). Both these assays display a high specificity and improve sensitivity for sperm DNA visualization (Fernández *et al.,* 2005; Thomson *et al.,* 2011).

1.12.2 Techniques to Determine Chromatin Packaging Quality in Spermatozoa

Immature chromatin condensation is another possible type of DNA damage in human spermatozoa. Chromatin of mature spermatozoa has been shown to possess a varying binding capacity for many nuclear dyes and stains. This binding capacity reflects anomalies in the chromatin packaging quality. Chromatin structural probes using nuclear dyes are easy to use. However, their cytochemical basis is rather complex. Several factors influence the staining of the sperm chromatin, such as the secondary structure of DNA, regularity and density of the chromatin packaging, and binding of DNA to chromatin proteins (Erenpreiss *et al.,* 2006). Therefore, tests have been developed for the evaluation of sperm DNA packaging and maturity. These tests include DNA fluorescence stains or fluorochromes, such as chromomycin A_3 (CMA_3) and aniline blue (AB) (Varghese *et al*., 2009).

- **Chromomycin A_3 (CMA_3) Staining Test**

CMA_3 is a guanine-cytosine-specific fluorochrome and competes with protamines for binding to the minor groove of the DNA helix, thus detecting protamine-deficiency in loosely packed chromatin. CMA_3 stains the post acrosomal part of the sperm head. The post acrosomal region of spermatozoa with immature chromatin packaging will be fluorescent yellow after staining. High levels of CMA_3 fluorescence are therefore indicative of a low protamination state. Dull or no fluorescent stain indicates mature chromatin packaging (Esterhuizen *et al.,* 2000; Agarwal *et al.,* 2004; Varghese *et al*., 2009).

- **Aniline Blue (AB) Staining Test**

AB staining is used to assess the nuclear maturity of spermatozoa. This test is especially helpful for the detection of extra lysine-rich histones. This may be an

indication of lower amounts of protamines in the sperm nucleus, as well as immature chromatin condensation. AB distinguishes between lysine-rich histones and protamines. Spermatozoa with immature chromatin condensation will stain positive blue, whereas spermatozoa containing mature chromatin will not be susceptible to the stain (Kazerooni *et al*., 2009).

1.12.3 Techniques to Determine Sperm Chromosomal Aneuploidies

Fluorescent *in-situ* hybridization (FISH) is useful for the detection of numerical chromosomal abnormalities in human spermatozoa. FISH techniques expand the field of male infertility diagnosis (Ribas-Maynou *et al.,* 2012). The rapid identification of chromosomal defects in spermatozoa may reduce the risk of transmitting genetic errors to early embryogenesis (Balasuriya *et al.,* 2011). The most common aneuploidies which can occur in the human embryo include Klinefelter's syndrome (XXY), Turner's syndrome (X), Patau's syndrome (trisomy 13), Edward's syndrome (trisomy 18), and Down's syndrome (trisomy 21).

Highly specific fluorochrome probes are designed to recognize certain chromosome loci during hybridization for the identification of chromosomal aberrations within the sperm nucleus. The fluorochrome-labelled region of the chromosome can be screened using fluorescence microscopy. Many human chromosome probes are available. Multicolour FISH for multiple chromosome aberration screening is also possible. This entails three to five different chromosome-specific probes being hybridized in parallel to the sample of spermatozoa (Henkel, 2007; Qiu *et al.,* 2012).

Recently, microarray comparative genomic hybridization (aCGH) has been identified for chromosomal analysis and embryo aneuploidy screening. Array-CGH has been recognized to be more reliable than FISH techniques, since FISH fail to detect 20-50% of abnormal embryos. Array-CGH proves a high sensitivity with an inaccuracy rate as low as 2% (Kroener *et al.,* 2012).

1.13 Prevention of Sperm DNA Damage

Advanced knowledge about the etiology of sperm DNA damage is required if we are to enhance measures for the detection of DNA damage in spermatozoa and consider strategies for promising its prevention (Aitken & Bennetts, 2007). Natural antioxidants and enzymes in the male reproductive tract are known to protect spermatozoa

against DNA damage caused by oxidative stress and ROS. Also, once protamines have bound to DNA, it may shield spermatozoa against ROS damage (Leduc *et al*., 2008). The pharmacological supplementation of vitamins, specifically vitamin E, A and C, and minerals, along with antioxidant treatment may improve overall sperm quality and reduce the incidence of DNA damage in spermatozoa (Paduch *et al.,* 2007).

In ART, sperm preparation techniques can be applied to reduce the percentage of DNA damage in a sperm sample and provide functional spermatozoa (Zini *et al.,* 1999). Frequently used sperm preparation techniques include the swim-up method and gradient centrifugation. It has been reported that motile spermatozoa had higher mitochondrial membrane potential, decreased levels of DNA fragmentation, and produced lower levels of ROS after gradient centrifugation. The swim-up method also revealed a significant decrease in the percentage of spermatozoa with damaged DNA. Therefore, both sperm preparation methods provide spermatozoa with a low percentage of DNA damage (Ricci *et al.,* 2009). Seminal plasma is a vital component in semen, providing antioxidants to spermatozoa. The washing and separation of spermatozoa from seminal plasma during semen processing may results in a pro-oxidant state and an increase in ROS production (Zini *et al.,* 1999). Consequently, the prepared sperm sample should be used for insemination as soon as possible.

1.14 Treatment Modalities for High Levels of Sperm DNA Damage

The development of ART procedures have been a fundamental milestone in the treatment of male-factor infertility. Novel techniques have now made it possible to effectively treat patients displaying high levels of sperm DNA damage (Paduch *et al.,* 2007). The PICSI (physiologic ICSI) technique can be used to select mature spermatozoa with normal nuclei. During *in vivo* fertilization hyaluronic acid (HA) has a vital function in selecting only the mature spermatozoa, which ultimately penetrate the zona pellucida and fertilize the oocyte (Parmegiani *et al.,* 2010). During PICSI, mature spermatozoa will bind permanently to HA *in vitro*. These spermatozoa have completed plasma membrane remodelling, cytoplasmic extrusion, and nuclear maturity. Therefore, the mature spermatozoa may have a normal protamine content and intact DNA. HA sperm selection for ICSI contribute to higher fertilization rates, as well as increased implantation and pregnancy rates (Nasr-Esfahani et al., 2008; Parmegiani *et al.,* 2010).

Intracytoplasmic morphologically selected sperm injection (IMSI) is based on the selection of normal nuclear spermatozoa without vacuoles, using computer-enhanced digital microscopy at high magnification (> x6,000). Only the highest quality spermatozoa are selected during IMSI. Motile sperm organelle morphology examination (MSOME) has been developed for sperm selection (American Society for Reproductive Medicine, 2010; Wilding *et al.,* 2011). MSOME characterized subtle sperm morphology features, such as abnormal head size, midpiece defects, and the presence of vacuoles. The presence of large nuclear vacuoles in motile spermatozoa has been positively correlated with sperm DNA fragmentation. IMSI selected spermatozoa yield better quality embryos and higher pregnancy rates (Vanderzwalmen *et al.,* 2008; Wilding *et al.,* 2011).

Spermatozoa showing apoptotic-like features may have remarkably high levels of DNA fragmentation. A primary feature of apoptosis is the externalization of the phospholipid phosphatidylserine (PS) that is present on the inner plasma membrane. PS has been identified on the surface of apoptotic spermatozoa (Rawe *et al.,* 2010). It has been discovered that PS exhibits a high affinity for the phospholipid-binding protein annexin V. Spermatozoa with externalized PS will bind to microbeads combined with annexin V. This discovery contributed to the development of magnetic activated cell sorting (MACS) with annexin V microbeads to eliminate dead and apoptotic-like spermatozoa before selecting individual spermatozoa for ICSI (De Fried and Denaday, 2010). During the MACS procedure, annexin V-positive spermatozoa will accumulate in the annexin V column when a strong magnetic field is applied. The non-apoptotic spermatozoa will travel through the column and are considered annexin V-negative. The selection of non-apoptotic spermatozoa for ICSI may increase pregnancy outcome (Rawe *et al.,* 2010).

Testicular and epididymal spermatozoa are suitable for ICSI. Studies have shown that epididymal spermatozoa have increased levels of DNA damage, as these spermatozoa have been reserved for an extended period. Testicular spermatozoa have lower levels of DNA fragmentation compared to epididymal spermatozoa (Steele *et al.,* 1999; Ramos *et al.,* 2004). Epididymal sperm aspiration and testicular sperm extraction procedures can be used to obtain spermatozoa. However, testicular biopsies are recommended, since a reduced amount of DNA damage is found in testicular spermatozoa (Steele *et al.,* 1999).

1.15 Correlation of Sperm DNA with Sperm Morphology

Many studies have confirmed a distinct association between sperm morphology and embryo development, implantation rates and pregnancy outcome. Spermatozoa may appear morphologically normal at high-power magnification, but its potential to fertilize a mature metaphase II oocyte is still not guaranteed. This could be because fertilization potential of human spermatozoa also depends on its chromatin condensation and DNA integrity.

Chromatin maturation during spermiogenesis is an important component of its fertilization potential. Sperm chromatin condensation does not play a direct role in the shaping of the sperm head, but protamine binding to DNA does result in the production of an uncharged chromatin complex that enables the DNA molecule to be condensed into a volume that is typically 10% or less than that of a somatic cell nucleus. This condensation results in a more hydrodynamic sperm head and contributes indirectly to the head shape. Sperm containing poor chromatin packaging frequently have enlarged or abnormal head shapes (Balhorn, 2007). Likewise, an association between sperm head abnormalities (amorphous, elongated or round heads) and chromosomal abnormalities have been observed (Chemes *et al.,* 2007).

Cassuto *et al.* (2009) also identified a link between DNA compaction and sperm head morphology. Oocytes microinjected with a spermatozoon, displaying abnormal head morphology with one or more vacuoles, did not develop into good-quality blastocysts. Varghese *et al*. (2009) concluded that normal sperm morphology and head defects were correlated with incidence of sperm DNA normality. The presence of large nuclear vacuoles in the sperm head may be associated with poor chromatin condensation. Chromatin condensation is important for the protection of the paternal genome, especially during genetic transfer from the male gamete to the oocyte before fertilization (Cassuto *et al.,* 2012).

Studies have been conducted to evaluate the relationship between sperm morphology and recorded IVF rates among men with normozoospermia and teratozoospermia. A highly negative correlation existed between percentage normal sperm morphology and poor chromatin packaging quality. A positive correlation was recorded for normal morphology and IVF rates (Esterhuizen *et al.,* 2000).

Previous results demonstrated absent DNA fragmentation in normal spermatozoa from fertile men and significantly higher levels in infertile men with moderate to severe teratozoospermia (Avendaño *et al.,* 2009; Tang *et al.,* 2010). Three morphological abnormalities in spermatozoa, that is broken necks, abnormal necks, and curled tails showed a positive correlation with fragmented sperm DNA during SCSA analysis (Cohen-Bacrie *et al.,* 2009).

1.16 Objectives of this Study

In this study the primary objective was to ascertain the prevalence of abnormal sperm DNA (in men visiting a fertility clinic) and association with sperm morphology, specifically in two sperm morphology groups [p-pattern (0-4%) and g-pattern (5-14%)], using different and separate sperm DNA tests.

Secondary objectives included: (i) correlation of sperm DNA outcome with fertilization *in vitro;* (ii) descriptive data on embryo quality with regards to sperm DNA; (iii) indication on pregnancy outcome with regards to sperm DNA, and (iv) identification of a suitable sperm DNA test for clinical diagnostic use in an ART program.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study Population and Semen Sample Collection

This prospective analytical study was conducted at the Vincent Pallotti Fertility Clinic, Cape Town, South Africa between May 2011 and October 2012. The Health Research Ethics Committee (HREC) of the University of Stellenbosch approved the study protocol (REF Number: N11/06/173). A total of 573 fresh semen samples from andrology (n=474), IUI, IVF and ICSI patients were collected. The ART samples were collected on the day of the procedure. Overall, 99 cycles (ART) were included, of which 47 cycles involved IUI, 21 cycles involved IVF and 31 cycles involved ICSI. The mean female age for the IUI subgroup was 36.7 years. A slightly lower mean female age of 34.8 years was identified in the IVF/ICSI subgroup.

Following semen liquefaction, a basic semen analysis (volume, pH, concentration, motility, and MAR-test) was performed according to World Health Organization (WHO, 2010) criteria. Semen smears for sperm morphology were prepared according to WHO guidelines (WHO, 2010) (Addendum I and II). Semen smears were also prepared for a standard CMA3 staining test to evaluate sperm chromatin packaging quality (Addendum I and III). For sperm DNA fragmentation assessment, semen samples were prepared using standard procedure for the TUNEL assay (Addendum IV).

TABLE 2.1

Study Design

Study Population and Outcomes Measured in each Subgroup	
Study Population	**Outcomes Measured**
Andrology Samples (n=474)	Sperm Morphology TUNEL CMA3
IUI Samples (n=47)	Sperm Morphology TUNEL CMA3 Pregnancy Rates
IVF Samples (n=21)	Sperm Morphology TUNEL CMA3 Fertilization Rates Embryo Quality Pregnancy Rates
ICSI Samples (n=31)	Sperm Morphology TUNEL CMA3 Fertilization Rates Embryo Quality Pregnancy Rates

2.2 Sperm Morphology Assessment

Sperm morphology was analyzed by optical light microscopy according to the Tygerberg Strict Criteria after the Diff-Quik staining method (Addendum II) was applied. The whole spermatozoon (head, neck, midpiece and tail) was considered during morphology evaluation. A spermatozoon was considered normal when the head exhibited a symmetrical oval shape, the acrosomal region comprised 40-70% of the sperm head, and the tail was straight without neck or midpiece defects (Kruger *et al.,* 1986; Menkveld *et al.,* 1990; Kruger *et al.,* 1996). Sperm morphology evaluation of each sample was analyzed by the principal investigator and at least one independent investigator from the Vincent Pallotti Fertility Clinic.

2.3 Chromomycin A_3 (CMA_3) Staining Test

Following preparation of smears, slides were fixed in methanol/acetic acid 3:1 at room temperature for 20 minutes and air-dried. Slides were stained with 30µl CMA_3 while kept in a dark chamber for 20 minutes. Slides were then rinsed in McIlvaine's buffer and mounted with Dabco anti-fade solution. An Olympus BX40 fluorescence microscope (Wirsam Scientific, Cape Town, South Africa), equipped with the appropriate filter and excitation of 465-495nm was used for slide evaluation (Esterhuizen *et al.,* 2000).

A total of 100 spermatozoa were randomly assessed for each patient slide. Spermatozoa with bright yellow staining (CMA_3 positive) were regarded as immature spermatozoa with decondensed chromatin. Dull yellow spermatozoa (CMA_3 negative) contained good chromatin packaging and were therefore regarded as mature spermatozoa. The chromatin packaging was considered normal for CMA_3 values ≤ 40%. Dubious abnormal chromatin packaging occurred when CMA_3 values were between 41–60%. Sperm chromatin packaging was considered abnormal for CMA_3 values ≥ 61% (Esterhuizen *et al.,* 2000). In 90% of cases in this study population the inter-technician variation for CMA_3 values was less than 11% (Addendum III).

2.4 TUNEL Assay

Sperm DNA fragmentation was assessed using the commercially available DeadEnd™ Fluorometric TUNEL System (Promega, Madison, USA). The assay uses

fluorescein-dUTP to label single- and double-stranded DNA breaks. Firstly, spermatozoa were washed by centrifugation at 300xg for 10 minutes in phosphate-buffered saline. Then 10-50µl of the sperm suspension was spread onto Starfrost slides and air-dried. Slides were fixed for 25 minutes in formaldehyde (final concentration 4%) and washed for 5 minutes in freshly prepared phosphate-buffered saline (Henkel *et al.,* 2004).

Spermatozoa were permeabilized with 0.2% Triton X-100 for 5 minutes and rinsed twice in phosphate-buffered saline. Then 100µl of equilibration buffer was added to each slide. After 5 minutes, 20µl of the prepared TdT-labelling solution was aliquoted onto the slides. Slides were immediately covered with plastic coverslips and incubated in a dark humidified chamber for 1 hour at 37ºC. Coverslips were carefully removed and slides were immersed in 2x SSC for 15 minutes in a dark chamber at room temperature to terminate the reaction. Finally, slides were rinsed twice in deionized water (Henkel *et al.,* 2004).

Slides were analyzed immediately. A total of 100 spermatozoa were randomly evaluated under a fluorescence microscope (Olympus BX40). Spermatozoa were classified according to intact or fragmented DNA. Spermatozoa showing no fluorescence had intact DNA (TUNEL negative), while green nuclear fluorescence indicated fragmented sperm DNA (TUNEL positive). Sperm DNA status was considered normal for TUNEL values ≤ 30%. TUNEL values > 30% indicated fragmented sperm DNA (Henkel *et al.,* 2004). The inter-rate reliability for the TUNEL assay has been established (Addendum IV).

2.5 Sperm Preparation for ART Cycles

Standardized methods were followed. The sperm preparation method was determined by the quality of the semen sample produced. Two basic methods, swim-up and gradient centrifugation, were used. Sperm preparation buffer (SAGE, Cooper Surgical, Ferring, South Africa) was used for wash procedures and SilSilect gradient preparation (FertiPro, Harrilabs, South Africa) for gradient centrifugation.

2.6 Fertilization Techniques

In vitro fertilization techniques were done according to standard routine methods and procedures of the Vincent Pallotti Fertility Clinic. For this study different fertilization methods were applied. These included IUI, IVF, and ICSI.

2.6.1 Intra-Uterine Insemination (IUI)

Oocyte production by the ovaries was stimulated by the use of fertility drugs. This step was carefully monitored to ensure that not too many oocytes developed. At the time of ovulation, a prepared sperm sample was injected into the uterus through the vagina, which brings the sperm and oocyte into close proximity (Montanaro Gauci *et al.,* 2001).

2.6.2 In Vitro Fertilization (IVF)

Mature metaphase II oocytes were inseminated with 100 000 to 500 000 motile spermatozoa and incubated in the embryo stage-specific mediums (SAGE, Cooper Surgical, Ferring, South Africa), using a routine standard IVF method (Drs Aevitas Fertility Clinic, Vincent Pallotti Hospital).

2.6.3 Intracytoplasmic Sperm Injection (ICSI)

Spermatozoa were immobilized in polyvinylpyrrolidone (PVP). Each mature metaphase II oocyte was injected with a single immobilized spermatozoon, using a routine standard ICSI method. Embryos were then incubated in the embryo stage-specific mediums (SAGE, Cooper Surgical, Ferring, South Africa) (Windt *et al.,* 2002).

2.7 Embryo Culture and Evaluation

The normal standardized routine method was used for all patients. After incubation for 16-18 hours the injected/inseminated oocytes were inspected for fertilization (presence of two pronuclei). During further embryo development (day 2, 3, 4 and 5 post-insemination) the embryo quality was evaluated, according to their blastomere morphology, mononucleation, percentage fragmentation and blastocyst formation. Embryos were incubated individually in drops and transferred daily to fresh cleavage

or blastocyst medium. Embryos were regarded as 'good quality' embryos when they were at the four-cell stage at 48 hours post-injection/insemination or at the six- to eight-cell stage, 72 hours post-injection/insemination with equal sized blastomeres and minor or no cytoplasmic fragmentation (Windt *et al.,* 2004).

2.8 Blastocyst Evaluation

Blastocysts were graded on the basis of their degree of expansion and hatching status. The development of an inner cell mass and the trophectoderm appearance were also evaluated (Gardener and Schoolcraft, 1999).

2.9 Embryo Transfer

A standardized method was followed. Viable day 3 or blastocyst stage embryos were transferred into the uterus (trans-vaginal transfer), using the Efficiere Series transfer catheter (Cooper Surgical). A maximum of three embryos per patient were transferred (Windt *et al.,* 2002).

2.10 Pregnancy Evaluation

In this study a pregnancy was reported as positive when serum βhCG (IU/mL) concentrations were more than 10 IU/mL on day 10 post insemination – all pregnancies reported were therefore biochemical pregnancies (Windt *et al.,* 2002). The Drs Aevitas Fertility Clinic at the Vincent Pallotti Hospital where the study was conducted is mainly a referral facility and clinical pregnancy and live birth outcome data is very difficult to obtain.

2.11 Statistical Analysis

Statistical analysis was carried out using the Stata version 12.0 software for Windows by Dr. CJ Lombard of the Biostatistics Unit, Medical Research Council. The Spearman's non-parametric correlation coefficient test was performed to determine the relationship between sperm morphology and sperm DNA quality, as measured in the two different DNA tests. Linear regression analysis and Pearson's correlation coefficient test were also applied to establish further correlation between sperm morphology, CMA_3 and TUNEL values. In the ART groups, fertilization, embryo quality and pregnancy outcome were also correlated to sperm morphology and each

DNA test outcome, using the binomial regression model, multiple regression model, as well as the odds ratio model. *P*-values < 0.05 were considered statistically significant.

CHAPTER 3

RESULTS

A brief overview of the different subgroup populations and the outcomes measured is presented in Table 3.1.

The study population within each subgroup (andrology, IUI, IVF/ICSI) was divided into one of two sperm morphology groups (p- and g-pattern), based on their percentage (%) normal sperm morphology.

The number of participants within each morphology group is indicated in Table 3.1. The mean % CMA3 positive spermatozoa, as determined by the CMA3 staining test, were higher in all subgroups when compared to g-pattern groups. A similar finding was observed for the mean % TUNEL positive spermatozoa, as evaluated using the TUNEL assay. P-pattern groups demonstrated higher % TUNEL positive spermatozoa (fragmented DNA), compared to g-pattern groups.

The mean percentages for the IVF/ICSI fertilization rates (70.3% vs. 79.2%) were higher in the g-pattern sperm morphology group, in comparison with the p-pattern group. Therefore, a trend between % normal morphology and fertilization rates can be described.

The mean percentage of good quality embryos for the IVF/ICSI subgroup (57.6% vs. 47.9%) was also higher in the g-pattern sperm morphology group, in contrast with the p-pattern group. Once again it seems as if a relationship between % normal morphology and embryo quality exists.

Pregnancy rates for the IVF/ICSI subgroup were higher in the g-pattern morphology group (12.5% vs. 29.5%). Pregnancy rates for the IUI subgroup were lower in the g-pattern group (22.2% vs. 15.8% respectively). This data is however only applicable to this study population and the effect of the sperm DNA status on fertilization rates, embryo quality and pregnancy rates will also be discussed as secondary objectives further on in this chapter.

TABLE 3.1 Summary of Outcomes Measured in the Different Subgroup Populations

Subgroup Population	Morphology Pattern	N	% CMA3 Positive	% TUNEL Positive	Fertilization Rates (%)	Embryo Quality (%)	Pregnancy Rates (%)
Andrology	P-pattern	52	54.5	42.5	NA	NA	NA
	G-pattern	422	19.5	29.1			
IUI	P-pattern	9	52.0	36.2	NA	NA	22.2
	G-pattern	38	38.7	24.6			15.8
IVF/ICSI	P-pattern	8	50.4	35.9	70.3	57.6	12.5
	G-pattern	44	39.5	22.4	79.2	47.9	29.5

3.1 Primary Objectives: Prevalence of Abnormal Sperm DNA in P-pattern and G-pattern Morphology Group Patients (n=573)

3.1.1 CMA3 Results

The correlation between sperm morphology and chromatin packaging quality, as determined by the CMA3 staining test, for the 573 patients is illustrated in Figure 3.1.

Non-Parametric Smoother

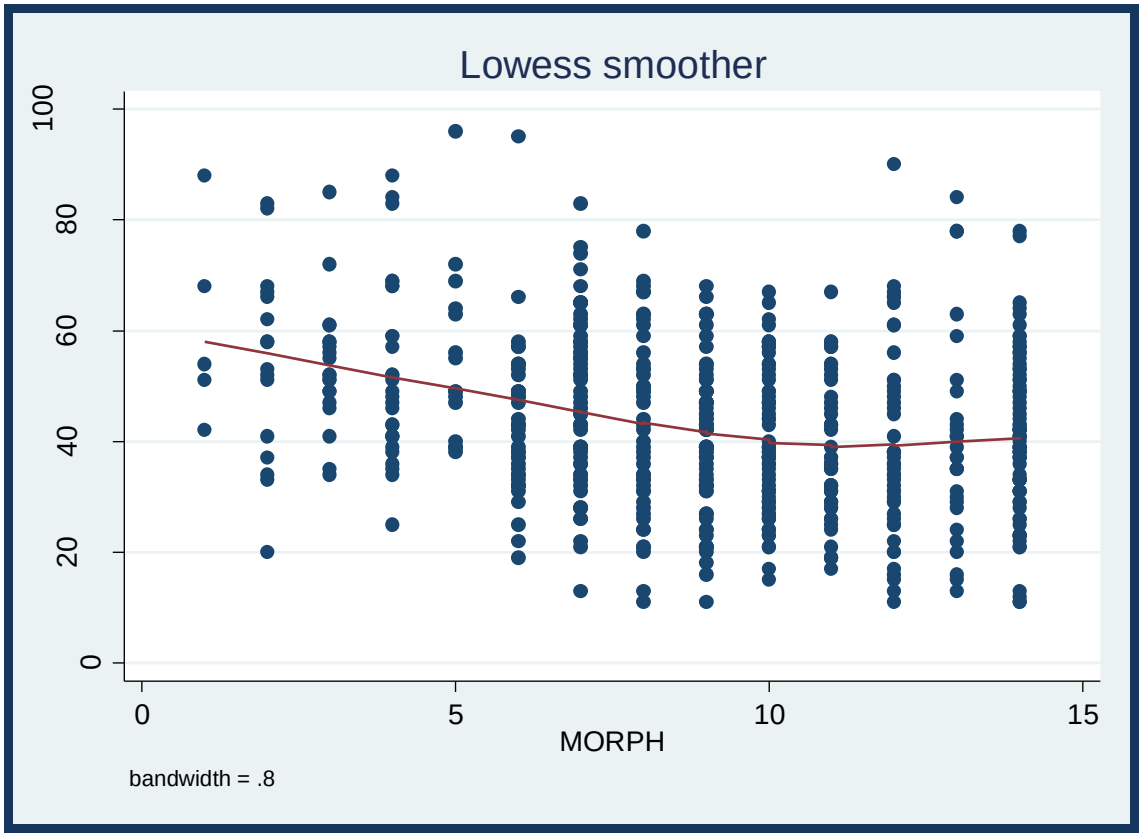

Figure 3.1: Lowess smoother graph showing the correlation between % normal sperm morphology and % CMA3 positive spermatozoa (r = - 0.26, *P* < 0.01).

Although a modest correlation between the CMA3 values and sperm morphology was observed, the association was significantly negative (r = - 0.26, *P* < 0.01). Increased CMA3 values were therefore associated with lower sperm morphology percentages. However, CMA3 was not associated with sperm morphology ≥ 10%, since there was no change in the mean CMA3 values in that range. A linear association was observed up to that point. A quadratic model was applied to test for a possible non-linear association between % CMA3 positive spermatozoa and % normal

morphology. The quadratic term was significant ($P < 0.0001$). Therefore CMA3 has a non-linear relationship with morphology in the range 0-14%. P-pattern and g-pattern morphology groups were relevant for CMA3 below 10% morphology – individual morphology percentages should be considered.

The differences in % CMA3 in the p- and g-pattern sperm morphology groups are presented in Figure 3.2

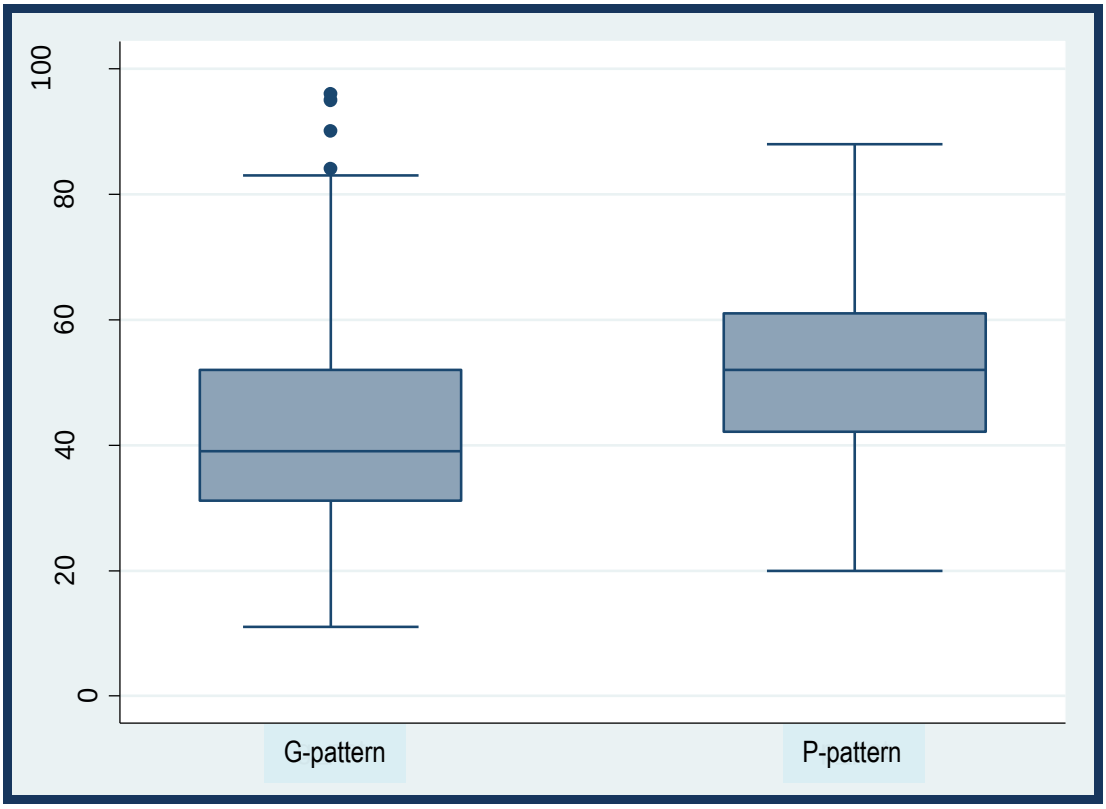

Figure 3.2: Box-and-whisker plot graph indicating median, minimum and maximum % CMA3 positive spermatozoa in the p- and g-pattern sperm morphology groups.

A two-sample t-test with unequal variances, showed a significant difference between the p-pattern and g-pattern morphology groups (53.7% [95% CI: 50.0-57.3] vs. 41.5% [95% CI: 40.3-42.9] $P < 0.01$) for % CMA3 positive spermatozoa. P-pattern spermatozoa had poorer DNA compaction compared to g-pattern spermatozoa.

The distribution of % CMA_3 in the p- and g-pattern sperm morphology groups is presented in Table 3.2.

TABLE 3.2

Comparison of number of patients with normal, dubious and abnormal CMA3 values according to p-pattern and g-pattern sperm morphology groups

Morphology Patterns	CMA3 Threshold Values			Total
	Normal (0-40%)	**Dubious** (41-60%)	**Abnormal** (61-100%)	
G-pattern	258	188	58	504
	51.19%	**37.30%**	**11.51%**	**100%**
P-pattern	12	38	19	69
	17.39%	**55.07%**	**27.54%**	**100%**

As seen in Table 3.2, 51.19% (258/504) of the study population in the g-pattern group revealed CMA3 values within the normal range (0-40% CMA3 positive spermatozoa); whereas 55.07% (38/69) of the study population in the p-pattern sperm morphology group showed dubious CMA3 values (41-60& CMA3 positive spermatozoa). One can therefore conclude that some spermatozoa with poor sperm morphology may still have acceptable chromatin packaging quality.

3.1.2 TUNEL Results

Results for the TUNEL values, as determined by the TUNEL assay for the 573 patients, showed a significant negative association with % normal morphology, even though the correlation was rather modest ($r = -0.42$, $P < 0.01$) (Figure 3.3).

Non-Parametric Smoother

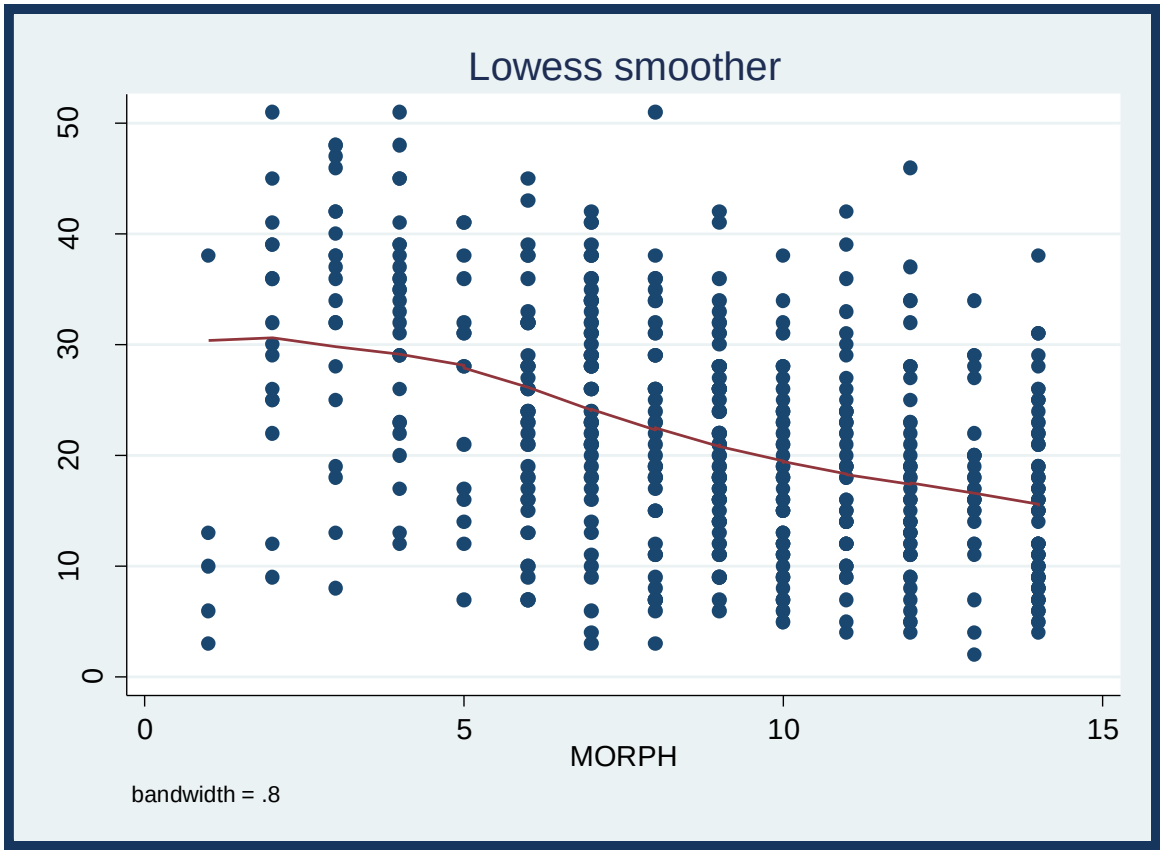

Figure 3.3: Lowess smoother graph showing the correlation between % normal morphology and % TUNEL positive spermatozoa (r = - 0.42, *P* < 0.01).

TUNEL values had a linear association over the range of sperm morphology, as depicted in Figure 3.3. Once again a quadratic model was applied to test for a non-linear association and the quadratic term showed no significance (*P* = 0.52). From this result we can therefore confirm that TUNEL has a linear association with sperm morphology. Therefore, also for TUNEL every level of sperm morphology matters and not only the p-patterns and g-patterns.

The differences in % TUNEL in the p- and g-pattern sperm morphology groups are presented in Figure 3.4

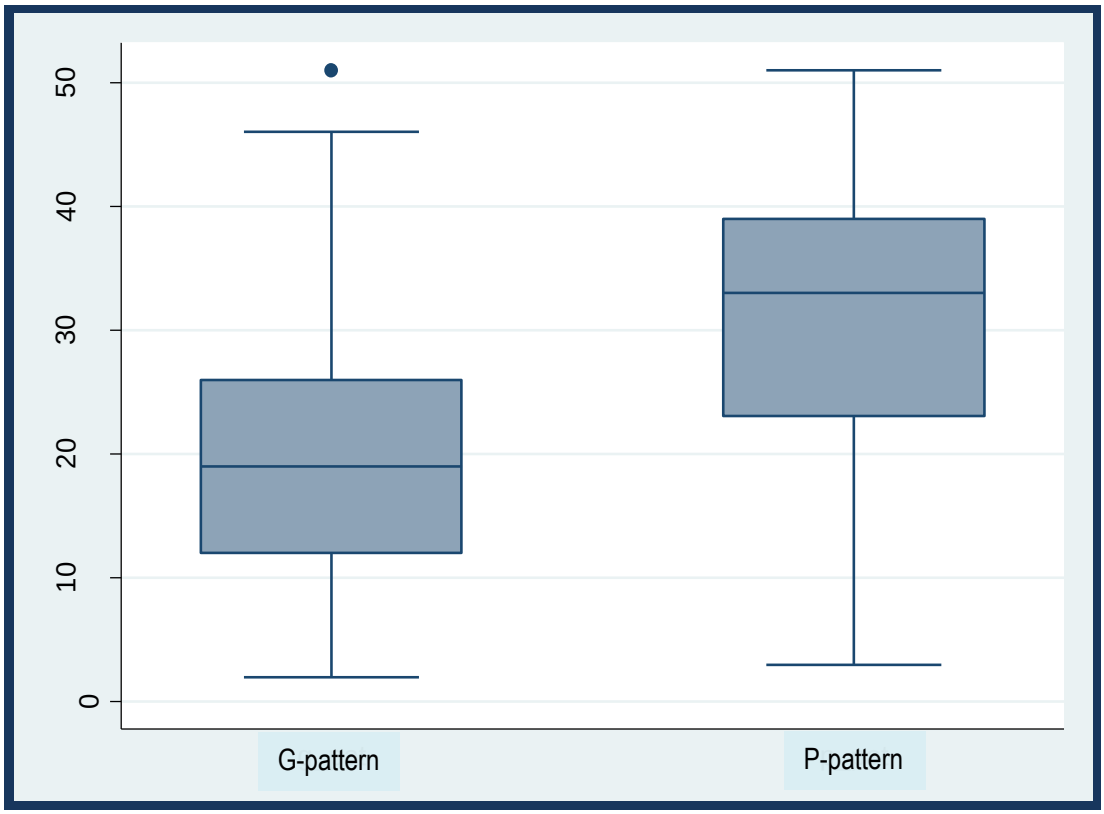

Figure 3.4: Box-and-whisker plot graph indicating median, minimum and maximum % TUNEL positive spermatozoa in the p- and g-pattern sperm morphology groups.

A two-sample t-test with unequal variances, showed a significant difference between the p-pattern and g-pattern morphology groups (31.0% [95% CI: 28.1-33.8] vs. 20.0% [95% CI: 19.1-20.7] $P < 0.01$) for % TUNEL positive spermatozoa. P-pattern spermatozoa had increased sperm DNA fragmentation compared to g-pattern spermatozoa.

3.1.3 Correlation of Different Sperm DNA Tests

The correlation between % CMA3 positive spermatozoa and % TUNEL positive spermatozoa, as determined by the CMA3 staining test and TUNEL assay respectively, are illustrated in Figure 3.5.

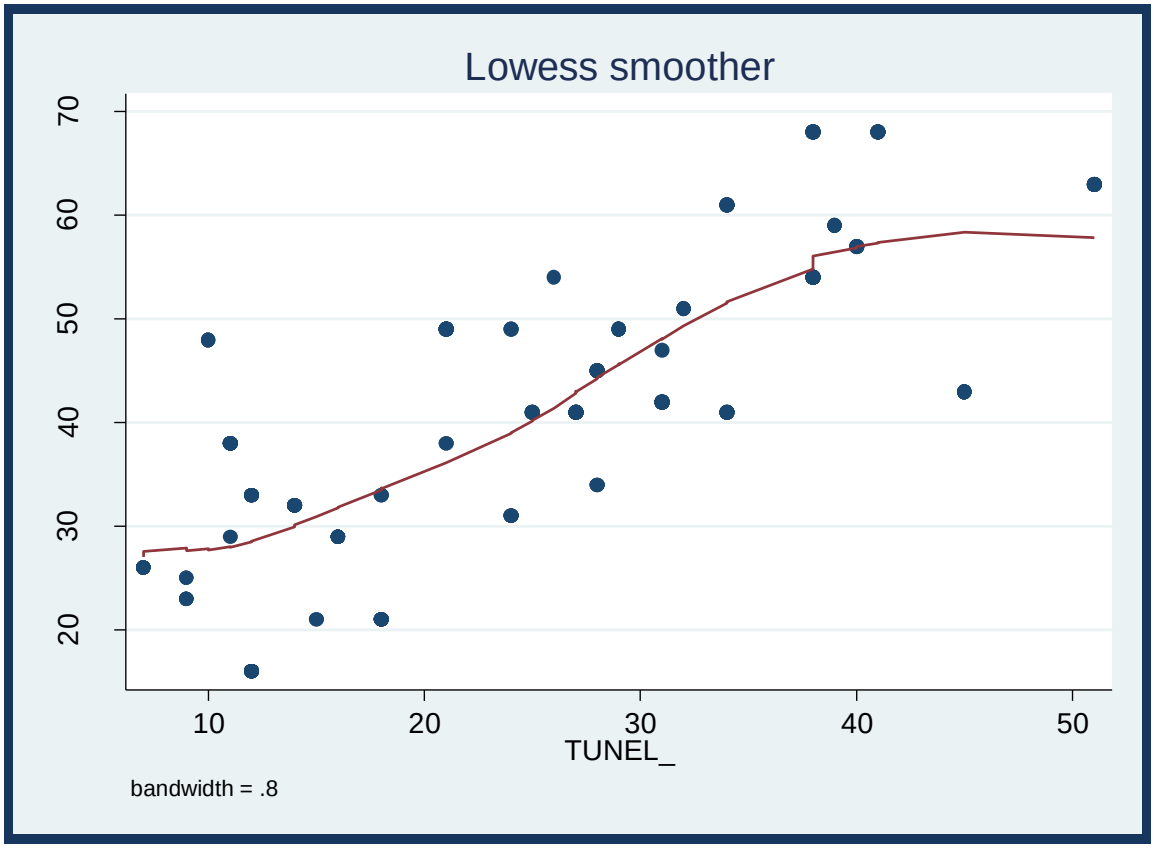

Figure 3.5: Lowess smoother graph showing the correlation between % CMA3 positive spermatozoa and % TUNEL positive spermatozoa (r = 0.36, *P* < 0.01).

Figure 3.5 shows a significant positive, but modest association between the % CMA3 positive spermatozoa and % TUNEL positive spermatozoa (r = 0.36, *P* < 0.01). This result indicates that there is a modest positive relationship between poor sperm DNA compaction and DNA fragmentation.

TABLE 3.3

Comparison of number of patients with normal, dubious and abnormal CMA3 values with number of patients with normal and abnormal TUNEL values

CMA3 Threshold Values	**TUNEL Threshold Values**		**Total**
	Normal (0-30%)	**Abnormal** (31-100%)	
Normal (0-40%)	**249**	**21**	**270**
	92.22%	7.78%	100%
	52.31%	17.95%	47.12%
Dubious (41-60%)	**153**	**73**	**226**
	67.70%	32.30%	100%
	33.55%	62.39%	39.44%
Abnormal 61-100%)	**54**	**23**	**77**
	70.13%	29.87%	100%
	11.84%	19.66%	13.44%
Total	**476**	**117**	**573**
	79.58%	20.42%	100%
	100%	100%	100%

Table 3.3 illustrates the number of patients with normal, dubious and abnormal CMA3 values compared with the number of patients with normal and abnormal TUNEL values. CMA3 values are indicated on the horizontal axis and these values are compared to the TUNEL values on the vertical axis. Of the normal CMA3 samples (270/573), 92.22% showed normal TUNEL values and 7.78% revealed abnormal TUNEL values. Of the normal TUNEL values (476/573), 52.31% showed normal CMA3 values, 33.55% displayed dubious CMA3 values and 11.84% indicated abnormal CMA3 values. This interpretation should be followed throughout the given Table 3.3.

Summary of Primary Objective Outcomes

- The % CMA3 positive spermatozoa have a significant negative, but modest non-linear relationship with % normal morphology.
- The % TUNEL positive spermatozoa have a significant negative, but modest linear relationship with % normal morphology.
- P-pattern and g-pattern morphology groups differ significantly from each other for % CMA3 positive spermatozoa. P-pattern has increased CMA3 values (poorer DNA compaction).
- P-pattern and g-pattern morphology groups differ significantly from each other for % TUNEL positive spermatozoa. P-pattern has increased TUNEL values (more DNA fragmentation).
- There is a significant positive, but modest association between the % CMA3 positive spermatozoa and % TUNEL positive spermatozoa.

3.2 Secondary Objectives: The Effect of Sperm Morphology and DNA Status on ART Outcome (n=99)

3.2.1 The Effect of Sperm Morphology and DNA Status on IUI Pregnancy Rates (n=47)

The correlation of semen parameters normal morphology, CMA_3 and TUNEL with biochemical IUI pregnancy outcome is presented in Table 3.4.

TABLE 3.4

Biochemical pregnancy rates reported for the 47 IUI patients compared to % normal morphology, % CMA3 positive spermatozoa and % TUNEL positive spermatozoa

	P-Values	
	Binomial Regression Model	**Multiple Regression Model**
Pregnancy Rates vs. % Normal Morphology	0.630	0.604
Pregnancy Rates vs. % CMA3 Positive	0.923	0.559
Pregnancy Rates vs. % TUNEL Positive	0.765	0.658

As seen in Table 3.4, there was no association between the three above mentioned factors (% normal morphology, % CMA3 positive spermatozoa and % TUNEL positive spermatozoa) and IUI pregnancy rates in this sample of patients ($P > 0.05$). See also Figure 3.6A, 3.6B, 3.6C.

The associations between % normal morphology, % CMA3 positive spermatozoa and % TUNEL positive spermatozoa and IUI pregnancy rates are demonstrated in Figures 3.6A, 3.6B and 3.6C respectively. The upper value (1) on the graphs is indicative of a positive pregnancy, whereas the lower value (0) indicates a negative pregnancy.

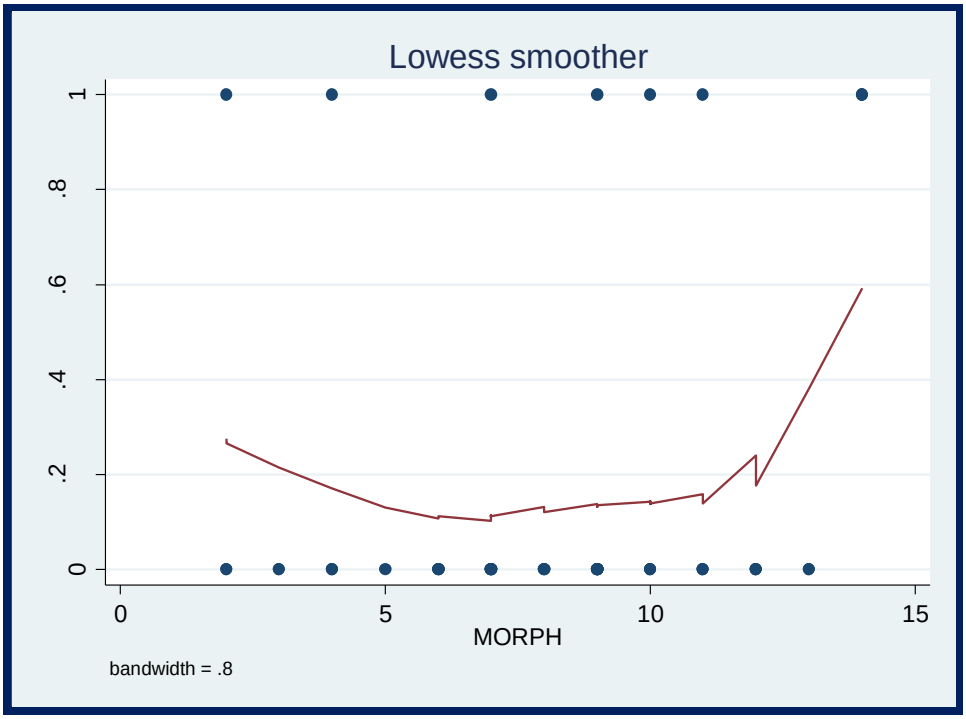

Figure 3.6A: Lowess smoother graph showing the effect of percentage normal morphology values on IUI biochemical pregnancy outcome [0 = not pregnant, 1 = pregnant].

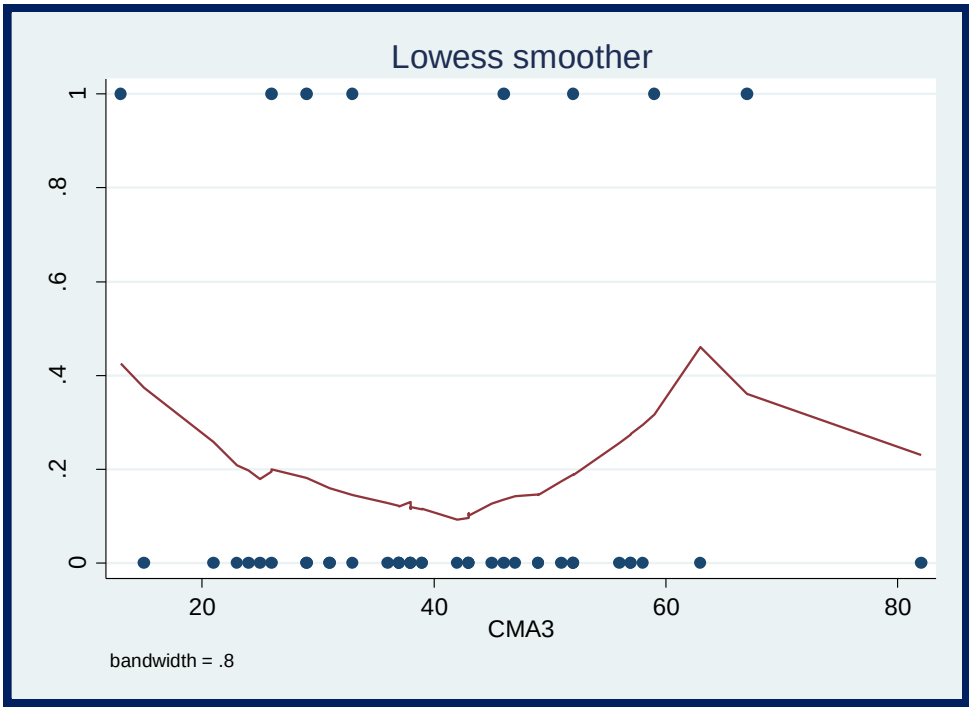

Figure 3.6B: Lowess smoother graph showing the effect of percentage CMA3 positive spermatozoa on IUI biochemical pregnancy outcome [0 = not pregnant, 1 = pregnant].

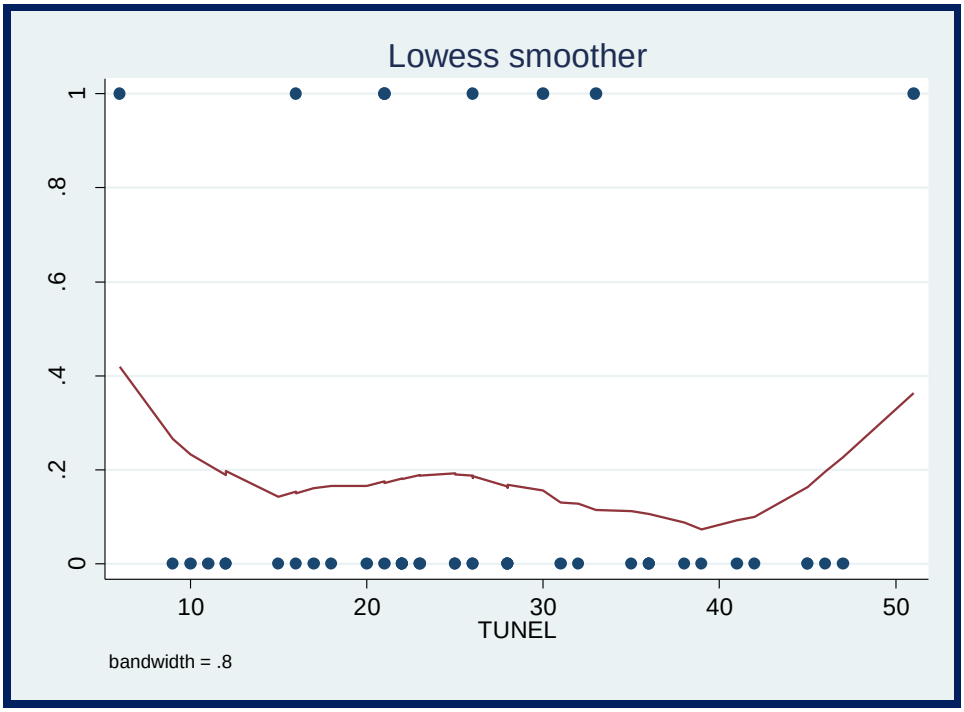

Figure 3.6C: Lowess smoother graph showing the effect of percentage TUNEL positive spermatozoa on IUI biochemical pregnancy outcome [0 = not pregnant, 1 = pregnant].

No trend was detected in any of the three graphs. Therefore, no association between % normal morphology, % CMA3 positive spermatozoa and % TUNEL positive spermatozoa and IUI pregnancy rates in this sample of patients could be observed (See Table 3.4).

3.2.2 The Effect of Sperm morphology and DNA Status on IVF/ICSI Pregnancy Rates (n=52)

Since the sample sizes for the IVF and ICSI subgroups were admittedly small, the two subgroups were combined and analysed together for higher numbers. This pooling of heterogeneous data revealed similar results when analysed separately.

In this study group of fifty-two IVF/ICSI couples 267 oocytes were obtained of which 79% (210/267) were fertilized and 52% (109/210) of the fertilized oocytes developed into good quality embryos. From the overall pool, 41% (109/267) of fertilized oocytes developed into good quality embryos.

The correlation of semen parameters normal morphology, CMA_3 and TUNEL with biochemical IVF/ICSI pregnancy outcome is presented in Table 3.5 and Figures 3.7.and 3.8.

TABLE 3.5

Biochemical pregnancy rates reported for the 52 IVF/ICSI couples compared to % normal morphology, % CMA3 positive spermatozoa and % TUNEL positive spermatozoa

	***P*-Values**	
	Odds Ratio Model	
Pregnancy Rates vs. % Normal Morphology	0.860	
Pregnancy Rates vs. % CMA3 Positive	0.022	0.041
Pregnancy Rates vs. % TUNEL Positive	0.044	

As seen in Table 3.5, there was no evidence of an association between % normal morphology and pregnancy rates ($P = 0.860$). The % CMA3 positive spermatozoa showed a significant association with pregnancy rates ($P < 0.05$). However, a U-shaped association (Figure 3.7, fractional polynomial model) was observed with pregnancies at the lower and upper CMA3 values. Therefore, two p-values have been reported (r = - 2.30, $P = 0.022$ and r = 2.04, $P = 0.041$ respectively). This was probably caused by the non-linear association of CMA3 and the pregnancies at the

high end of CMA3, which is unexpected – can be seen as outliers (comment by statistician). These outliers and the sample size can create problems for a categorical model. A significant negative association was observed between % TUNEL positive spermatozoa and pregnancy rates (r = - 2.01, *P* = 0.044). This association was linear (Figure 3.8). From this result we concluded that the likelihood of a pregnancy decreases by 6% for every percentage increase in the TUNEL value.

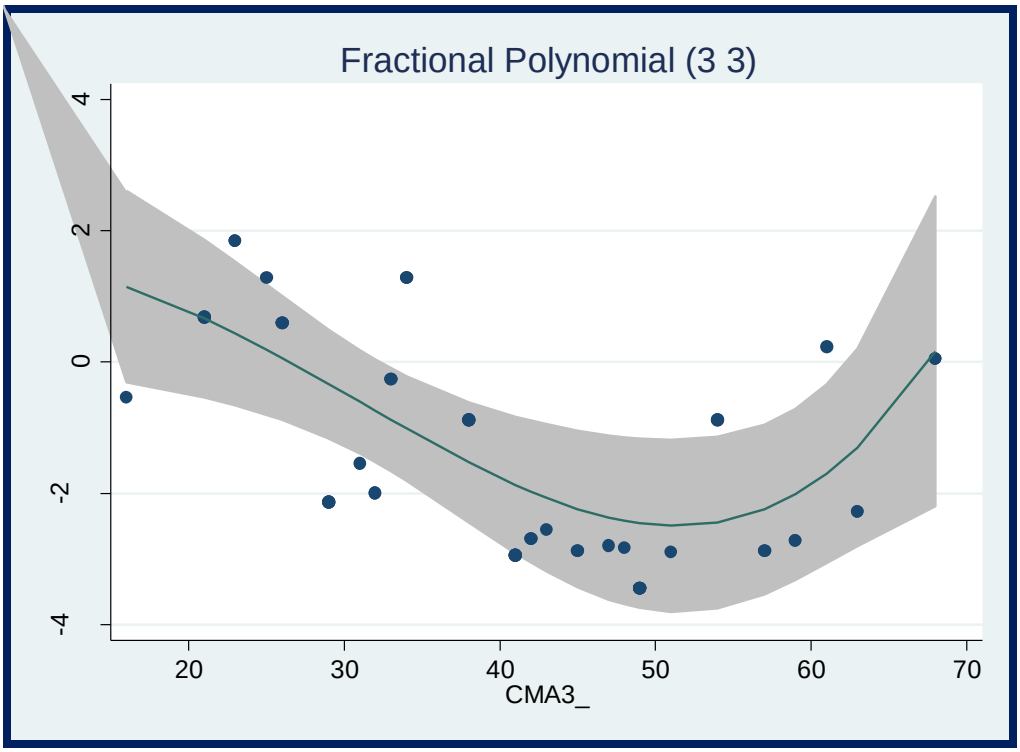

Figure 3.7: Fractional polynomial graph showing the association between % CMA3 positive spermatozoa and IVF/ICSI pregnancy rates (r = - 2.30, *P* = 0.022 and r = 2.04, *P* = 0.041).

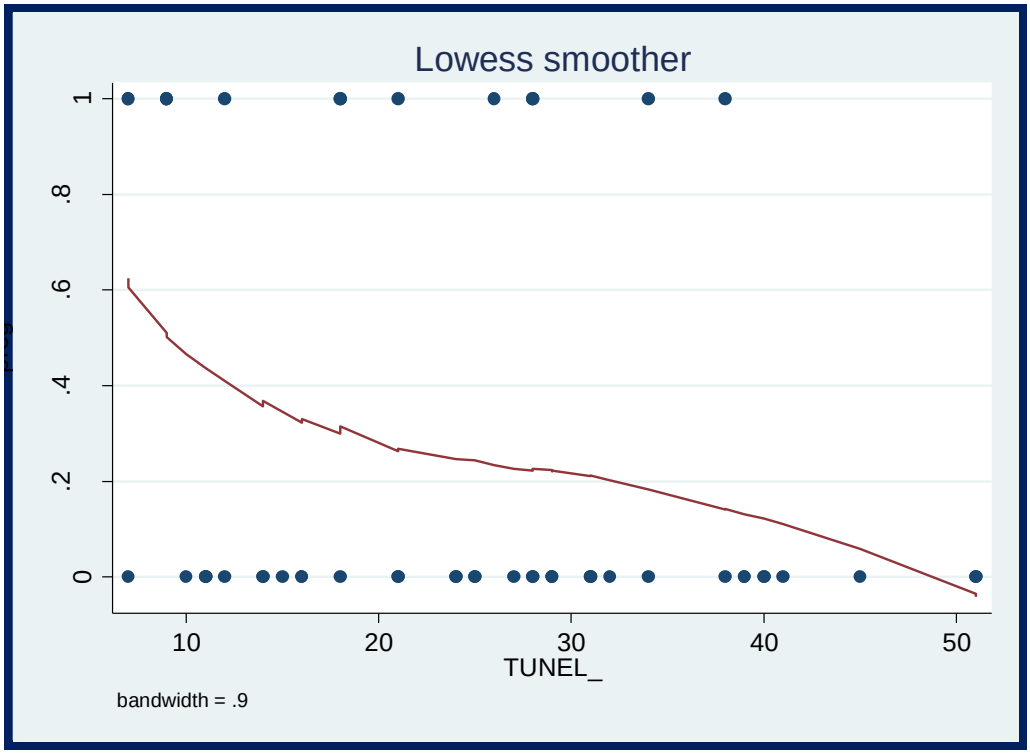

Figure 3.8: Lowess smoother graph showing the linear association between % TUNEL positive spermatozoa and IVF/ICSI pregnancy rates (r = - 2.01, *P* = 0.044) [0 = not pregnant, 1 = pregnant].

3.2.3 The Association between Procedure (IVF/ICSI) and Fertilization and Embryo Quality

The association between IVF and ICSI procedure and fertilization and embryo quality is presented in Table 3.6.

TABLE 3.6

The association between ART fertilization method (IVF/ICSI) and fertilization and embryo quality

Procedure	Fertilization		Embryo Quality		Total
	Negative	Positive	Poor	Good	
ICSI	43	110	102	51	153
	28.10%	**71.90%**	**66.67%**	**33.33%**	**100%**
IVF	14	100	56	58	114
	12.28%	**87.72%**	**49.12%**	**50.88%**	**100%**

In this sample of patients there was a clear association between the type of procedure (IVF/ICSI) and fertilization and embryo quality, as depicted in Table 3.6 Couples who underwent IVF cycles had higher fertilization rates (87.72% vs. 71.90%) and revealed better embryo quality (50.88% vs. 33.33%) compared to ICSI cycle patients.

It was therefore thought that the method of fertilization (IVF or ICSI) could impact on results and for the following statistical analysis, type of procedure was incorporated.

3.2.4 The Association between IVF/ICSI Fertilization Rates and Sperm Morphology and DNA Status (n=52)

The correlation of ART (IVF/ICSI) fertilization rates with normal sperm morphology, CMA_3 and TUNEL is presented in Table 3.7 and Figures 3.9 A, 3.9B and 3.9C.

TABLE 3.7

Fertilization rates reported for the 52 IVF/ICSI couples compared to % normal morphology, % CMA_3 positive spermatozoa and % TUNEL positive spermatozoa by incorporating the type of procedure (IVF or ICSI)

	***P*-Values**	
	Binomial Regression Model	**Odds Ratio Model**
Fertilization Rates vs. % Normal Morphology	0.948	0.860
Fertilization Rates vs. % CMA_3 Positive	0.602	0.348
Fertilization Rates vs. % TUNEL Positive	0.860	0.756
Fertilization Rates vs. No. of MII Ova	0.113	0.132
Fertilization Rates vs. Procedure	0.029	0.036

Both models in Table 3.7 showed that none of the three lab variables (% normal morphology, % CMA_3 positive spermatozoa and % TUNEL positive spermatozoa) were associated with fertilization rates, when the type of procedure was taken into consideration. See also Figures 3.9A, 3.9B and 3.9C. According to both models, the IVF procedure was however significantly associated with fertilization rates (P = 0.029 and 0.036) with IVF giving better fertilization than ICSI (Table3.7).

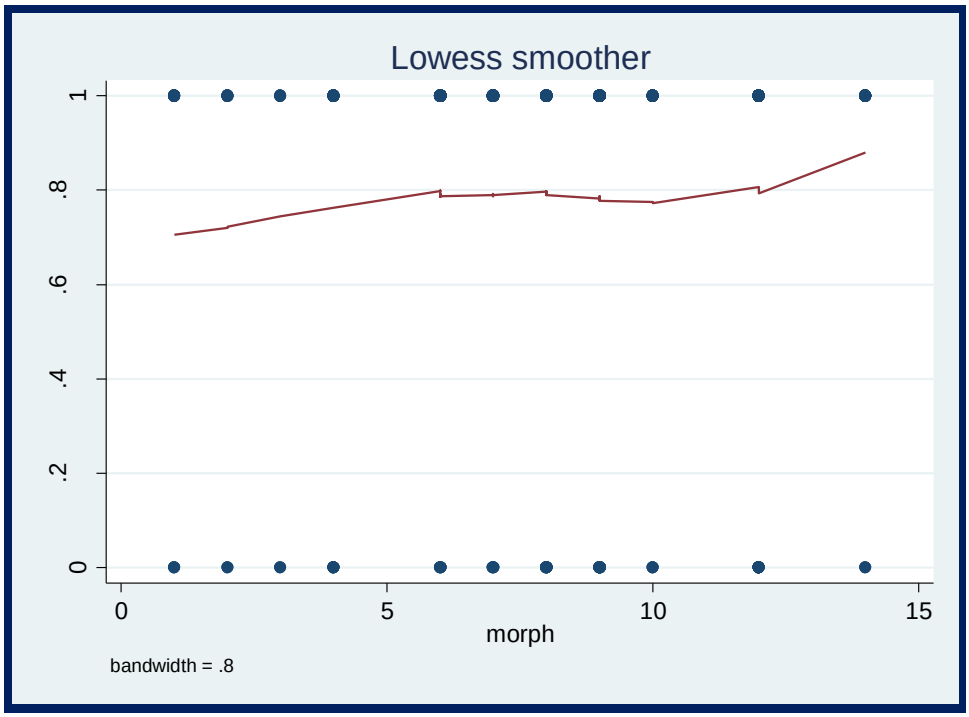

Figure 3.9A: Lowess smoother graph showing the effect of percentage normal sperm morphology on IVF/ICSI fertilization [0 = not fertilized, 1 = fertilized].

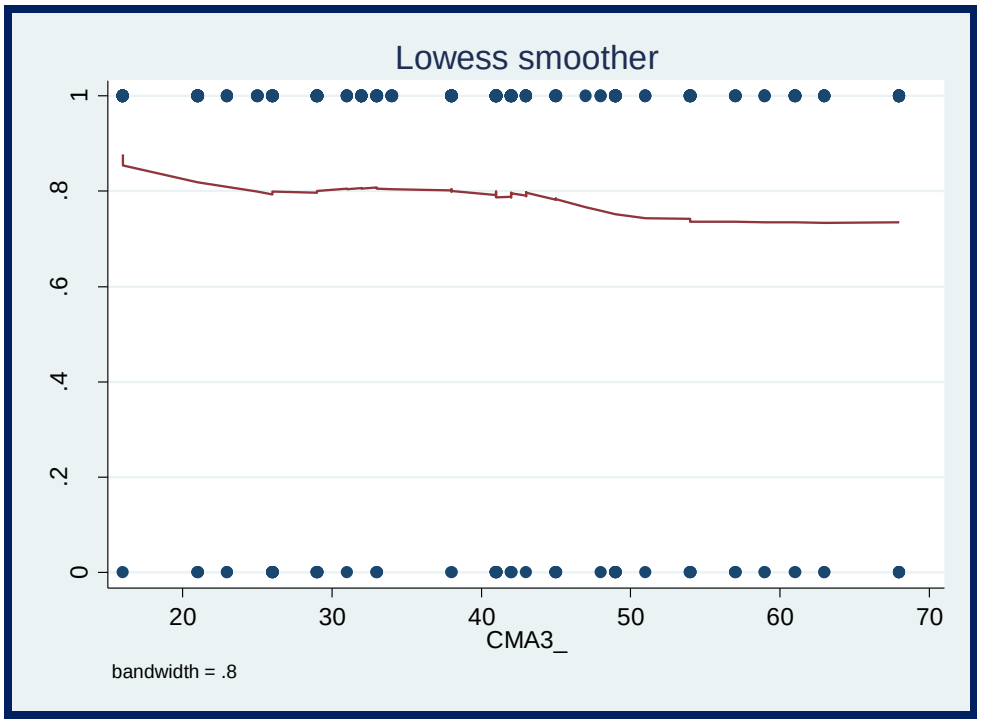

Figure 3.9B: Lowess smoother graph showing the effect of percentage CMA_3 positive sperm on IVF/ICSI fertilization [0 = not fertilized, 1 = fertilized].

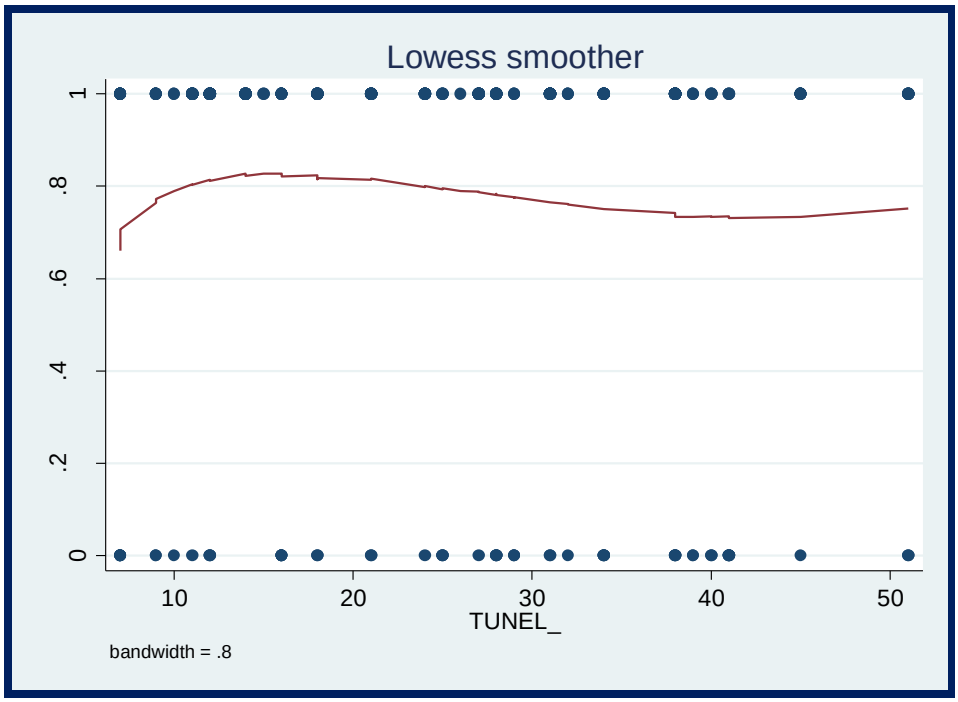

Figure 3.9C: Lowess smoother graph showing the effect of percentage TUNEL positive spermatozoa on IVF/ICSI fertilization [0 = not fertilized, 1 = fertilized].

The associations between % normal morphology, % CMA3 positive spermatozoa and % TUNEL positive spermatozoa and IVF/ICSI fertilization rates are revealed in Figures 3.9A, 3.9B and 3.9C respectively. The upper value (1) on the graphs is indicative of fertilization and the lower value (0) shows no fertilization. From this dataset no association between % normal morphology, % CMA3 positive spermatozoa and % TUNEL positive spermatozoa and IVF/ICSI fertilization rates was established (See Table 3.7).

3.2.5 The Association between IVF/ICSI Embryo Quality and Sperm Morphology and DNA Status (n=52)

The association between ART fertilization method (IVF/ICSI) and fertilization and embryo quality is presented in Table 3.8.

TABLE 3.8

Embryo quality reported for the 52 IVF/ICSI couples compared to % normal morphology, % CMA3 positive spermatozoa and % TUNEL positive spermatozoa by incorporating the type of procedure (IVF or ICSI)

	P-Values	
	Binomial Regression Model	**Odds Ratio Model**
Embryo Quality vs. % Normal Morphology	0.021	0.017
Embryo Quality vs. % CMA3 Positive	0.031	0.007
Embryo Quality vs. % TUNEL Positive	0.074	0.033
Embryo Quality vs. No. of MII Ova	0.086	0.083
Embryo Quality vs. No. of Fertilized Ova	0.041	0.036
Embryo Quality vs. Procedure	0.841	0.838

Table 3.8 shows the association between the three laboratory factors (% normal morphology, % CMA3 positive spermatozoa and % TUNEL positive spermatozoa) and embryo quality, as analysed using the binomial regression model. In the binomial regression model % normal morphology and % CMA3 positive spermatozoa were significantly associated with embryo quality (r = 2.31, *P* = 0.021 and r = 2.16, *P* = 0.031 respectively). Both were positively associated. The % normal morphology (r = 2.38, *P* = 0.017) and % CMA3 positive spermatozoa (r = 2.69, *P* = 0.007) were also significantly (positive) associated with embryo quality in the odds ratio model. The % TUNEL positive spermatozoa (r = - 2.13, *P* = 0.033) were significantly

(negative) associated with embryo quality. Therefore, poor sperm morphology and high TUNEL values were associated with poor embryo quality. Increased CMA3 was however associated with better embryo quality – an unexpected result. In both models the type of procedure was not associated with embryo quality (*P* = 0.841 and 0.838).

Summary of Secondary Objective Outcomes

- There was no association between % normal morphology, % CMA3 positive spermatozoa and % TUNEL positive spermatozoa and IUI pregnancy rates.
- There was no association between % normal morphology and IVF/ICSI pregnancy rates.
- There was a significant negative, but moderate linear association between % TUNEL positive spermatozoa and IVF/ICSI pregnancy rates.
- There was a significant positive (unexpected) u-shaped association between % CMA3 positive spermatozoa and IVF/ICSI pregnancy rates.
- There was no association between % normal morphology, % CMA3 positive spermatozoa and % TUNEL positive spermatozoa and IVF/ICSI fertilization rates.
- There was a significant positive association between % normal morphology and IVF/ICSI embryo quality.
- There was a significant positive association between % CMA3 positive spermatozoa and IVF/ICSI embryo quality (unexpected).
- There was a significant negative association between % TUNEL positive spermatozoa and IVF/ICSI embryo quality.

CHAPTER 4

DISCUSSION AND CONCLUSION

In recent years, significant progress has been made in the understanding of human fertilization *in vitro* and the vital role of the DNA integrity in human spermatozoa (De la Calle *et al.,* 2008). Various studies confirmed that male fertility potential may be affected by the sperm DNA quality. The use of spermatozoa with damaged DNA during ART procedures may also contribute to poor embryonic growth, as well as the transmission of abnormal genomic material to the progeny (Zhang *et al.,* 2010). Conventional semen parameters (sperm count, motility and morphology) do not display the DNA anomalies that occur at the genomic level (Kazerooni *et al*., 2009). The DNA integrity of human spermatozoa has shown potential as an important sperm parameter for male infertility assessment, in addition to conventional semen parameters (Irvine *et al.,* 2000; Simon *et al.,* 2011). Assessment of the sperm DNA integrity is valuable for the detection of male factors that may influence ART outcome (Shafik *et al*., 2006).

The primary objectives of the present study were to ascertain the prevalence of abnormal sperm DNA in p-pattern and g-pattern morphology group patients, as well as the association between sperm morphology and sperm DNA quality. The CMA3 staining test was applied to assess the DNA compaction and maturity in spermatozoa and the TUNEL for sperm fragmentation.

The percentage CMA3 positive spermatozoa for the 573 patients revealed a significant negative association with normal sperm morphology. We can therefore conclude that increased CMA3 values (less mature sperm) were associated with lower sperm morphology percentages although the association was only moderate. The relationship was non-linear in the range 0-14% and showed that CMA3 values were not associated with sperm morphology ≥ 10%. Therefore, p-pattern and g-pattern morphology groups were only relevant for CMA3 below 10% morphology. For interpretation we emphasize that individual morphology percentages should be taken into consideration and not only the broad p-pattern and g-pattern morphology groups. From the data it is clear that the p-pattern group included a range of CMA3 values. In this group there are therefore individuals with normal chromatin packaging. This is also true for the g-pattern group - there are individuals with good morphology,

but poor chromatin packaging. It would be of clinical importance to investigate the reproductive potential of these subgroups to identify which factor, chromatin packaging or sperm morphology play the biggest role. Comparative analysis for the two groups based on the CMA3 median values however, showed a significant difference between the p-pattern and g-pattern morphology group patients. Therefore, p-pattern spermatozoa, in general had poorer chromatin packaging quality compared to g-pattern spermatozoa. These results were supported by data from previous studies. Esterhuizen and colleagues confirmed a significant negative correlation between sperm morphology and CMA3 values (Esterhuizen *et al.,* 2000). A strong negative correlation between sperm morphology and the percentage of CMA3 positive spermatozoa was also described by Kazerooni *et al*. (2009). From these results we can therefore conclude that CMA3 testing should be done for all men and maybe especially for the g-pattern patients to identify the possibility of an additional factor in their infertility.

In the present study DNA fragmentation in spermatozoa was measured using the TUNEL assay. Results for the percentage TUNEL positive spermatozoa also showed a significant negative association with normal sperm morphology. The association was linear over the whole range of sperm morphology. Therefore, also for TUNEL every level of sperm morphology is important and not only the p-patterns and g-patterns. For TUNEL it is also evident that the p-pattern group included a range of values. In this group there are therefore individuals with normal sperm DNA fragmentation. This is also true for the g-pattern group - there are individuals with good morphology, but abnormal levels of sperm DNA fragmentation. Once again it would be interesting to look at the reproductive potential of these subgroups within the p-pattern and g-pattern groups. Comparative analysis for the two groups based on the TUNEL median values showed a significant difference between the p-pattern and g-pattern morphology groups. We concluded from our results that p-pattern spermatozoa had increased sperm DNA fragmentation compared to g-pattern spermatozoa. Similar results were confirmed by Sun *et al.* (1997) who established a negative correlation between the percentage of sperm DNA fragmentation, as detected by the TUNEL assay, and the morphology of ejaculated spermatozoa. Perrin *et al.* (2011) also found significantly higher levels of DNA fragmentation in patients with teratozoospermia (≤ 4% morphology), compared to the fertile control group. Results from the present study is however in contrast with the findings of Henkel *et al.* (2010) who did not observe an association between the two sperm

morphology groups (p-pattern and g-pattern) and DNA fragmentation, as recorded by the TUNEL assay.

The correlation between the two different sperm DNA tests (CMA3 and TUNEL) used during the current study was significantly positive, but modest. As a result, a modest positive relationship between DNA compaction and DNA fragmentation in spermatozoa can be described. Spermatozoa with immature chromatin packaging may therefore also have an increased percentage of DNA strand breaks.

As a secondary objective of this study we investigated the effect of sperm morphology and DNA status on ART (IUI, IVF and ICSI) outcome.

Pregnancy rates for the 47 IUI patients showed no association with normal sperm morphology, percentage CMA3 positive spermatozoa and percentage TUNEL positive spermatozoa. Our result is however only applicable to this group of patients. Earlier studies reported that the DNA damage in spermatozoa was significantly higher in couples who failed to achieve a pregnancy after IUI (Duran *et al.,* 2002; Bungum *et al.,* 2004; Bungum *et al.,* 2007; Schulte *et al.,* 2010). We do not have an explanation for this contradictory result other than the small size of the study population.

For the 52 IVF/ICSI cycles there were also no association between normal sperm morphology and pregnancy rates. The percentage CMA3 positive spermatozoa however showed a significant association with IVF/ICSI pregnancy rates. However, an unexpected u-shaped association was observed with sections that was positively and others that was negatively associated. The association was non-linear with pregnancies at the high end of CMA3, which is unexpected. This phenomenon may be the result of outliers. It is also possible that these patients are a subgroup that would be interesting to investigate. A significant negative association was observed between the percentage TUNEL positive spermatozoa and IVF/ICSI pregnancy rates. From this result we concluded that the likelihood of a pregnancy decreased by 6% for every percentage increase in the TUNEL value. Thomson *et al.* (2011) determined in his study that sperm DNA damage, as evaluated using the TUNEL assay, can be used to predict pregnancy outcome after IUI cycles, but not for ICSI cycles. According to Meseguer *et al.* (2011) the chance of achieving a viable pregnancy was low with sperm samples containing more than 30% DNA fragmentation.

In our study we also investigated the association between procedure (IVF/ICSI) and fertilization and embryo quality. In our sample of 52 patients there was a clear association between the procedure (IVF/ICSI) and fertilization and embryo quality. Couples who underwent IVF cycles had higher fertilization rates and revealed better embryo quality compared to ICSI cycle patients. This result could be explained by the fact that sperm morphology is used to determine fertilization procedure (IVF or ICSI) in the Vincent Pallotti Fertility Clinic. IVF cycles are therefore only done with excellent sperm samples, whereas ICSI cycles include many patients with abnormal semen profiles.

It was thought that the fertilization method (IVF/ICSI) may influence our results and therefore we incorporated the type of procedure into further statistical analysis of our data.

None of the three variables (normal sperm morphology, percentage CMA3 positive spermatozoa and percentage TUNEL positive spermatozoa) were associated with fertilization rates, when the type of procedure was incorporated. However, the IVF procedure was significantly associated with fertilization rates. This result was confirmed by two statistical models (binomial regression and odds ratio). According to the literature, the impact of sperm morphology on fertilization rates is rather controversial. De Vos *et al.* (2003) found that abnormal sperm morphology resulted in lower fertilization rates after ICSI. On the other hand, other studies indicated that fertilization rates achieved after ICSI with semen samples displaying severe teratozoospermia (≤ 4% morphology) did not differ from those with better overall sperm morphology (Raja and Franken, 2006). With regards to sperm DNA, Simon and Lewis (2011) found that fertilization rates correlated significantly with DNA fragmentation after IVF. Bakos *et al.* (2008) also established a significant negative correlation between IVF fertilization rates and DNA damage, using the TUNEL assay. In a study conducted by Nasr-Esfahani *et al.* (2005) CMA3 showed significant correlation with fertilization rates after ICSI.

Our finding can be explained by the fact that the paternal genome is not effective in the human embryo until day 3 (four- to eight-cell stage) and therefore the sperm effect was not yet evident. In follow up studies it would be interesting to rather look at blastulation rate.

During our investigation of the association between IVF/ICSI embryo quality and sperm morphology and DNA status (CMA3 and TUNEL) we found that normal morphology and percentage CMA3 positive spermatozoa were significantly (positive) associated with embryo quality. The percentage TUNEL positive spermatozoa were significantly (negative) associated with embryo quality. Therefore, poor sperm morphology and high levels of DNA fragmentation (TUNEL) were associated with poor embryo quality. Increased CMA3 was however associated with better embryo quality, which was an unexpected result and one that we have no feasible explanation for. The type of procedure was not associated with embryo quality. In a previous study conducted by Avendaño *et al.* (2010) a significant negative correlation was found between the percentage of DNA fragmentation in spermatozoa and embryo quality. We furthermore established in our results that the chance of having a good quality embryo was increased by a relative 15% for every percentage increase in sperm morphology. For every percentage increase in the TUNEL value the likelihood of having good quality embryos decreased by a relative 4%. Therefore, based on our results sperm DNA plays a small role in producing good quality embryos compared to sperm morphology.

In conclusion, sperm DNA anomalies may be different in type and degree and spermatozoa from some patients may be more susceptible to DNA damage than others. In many cases sperm morphology cannot be used to accurately predict the DNA quality of spermatozoa. Thorough evaluation is required for couples showing high levels of sperm DNA damage, low implantation rates, and poor embryo quality during ART procedures. Keeping this into consideration, the ultimate aim is to discover a sperm biomarker with the ability to predict male fertility status (Muratori *et al.,* 2010). Sperm DNA integrity has been considered in previous studies as an important biomarker for ART outcomes, especially for the prediction of fertilization rates, embryo quality and pregnancy outcome (Simon *et al*., 2011). However, many sperm DNA tests are currently utilized for research purposes only. The CMA3 staining test and TUNEL assay has a limited ability to distinguish between the p-pattern and g-pattern morphology groups. P-pattern spermatozoa are more likely to possess poor chromatin compaction and show increased levels of DNA fragmentation, but some p-pattern patients also may have normal DNA and g-pattern patients abnormal DNA.

Because of many controversies in literature, more investigations on the diagnosis of sperm DNA damage and the impact of DNA damage on assisted reproductive

outcome are needed. The universal standardization of sperm DNA protocols is necessary and male infertility parameters should be re-examined. It is recommended that a sperm DNA test should be implemented routinely in andrology laboratories for the clinical diagnosis of sperm DNA damage in patients.

ADDENDA

ADDENDUM I

PREPARATION OF SEMEN SMEARS

Method

1. Clean both sides of the frosted slide with lint-free tissue paper.
2. Label the slide at the frosted portion.
3. Place a 5–10µl semen droplet at the end of the slide, taking the sperm concentration into account.
4. Make a thin evenly spread smear, using a second slide to pull the semen droplet along the surface of the slide.
5. Allow the slide to air-dry.

The quality of the smear depends on:

- The sperm concentration (dilution factor: 1/10, 1/20, 1/100);
- The angle of the dragging slide (angle of slide: 45º, 30º, 10º).

ADDENDUM II

DIFF-QUIK STAINING METHOD

Materials

Hemacolor® Staining Set for Microscopy

Hemacolor® solution 1, fixing solution

Hemacolor® solution 2, colour reagent red

Hemacolor® solution 3, colour reagent blue

Method

Diff-Quik Staining:

1. Prepare smears, and allow air-drying.
2. Fix smears for 10 seconds in solution 1 fixative.
3. Stain slides for 7 seconds in solution 2.
4. Stain slides for 10 seconds in solution 3.
5. Rinse slides in water to remove excessive stain.
6. Allow slides to air-dry.

Evaluation:

7. Slides are evaluated using a light microscope.
8. Find a field of spermatozoa under 10 x magnification (brightfield microscopy).
9. Use immersion oil and change to the 100 x magnification (brightfield microscopy).
10. Count one hundred spermatozoa and evaluate their morphology.

ADDENDUM III

CHROMOMYCIN A_3 (CMA_3) STAINING TEST

Materials

McIlvaine's Buffer Stock Solutions

Solution 1: Dissolve 3.8424g citric acid (anhydrous) in 200ml distilled water

Solution 2: Dissolve 25.5564g disodium phosphate (anhydrous) in 900ml distilled water

McIlvaine's Buffer Working Solution (1L)

Dilute 176.5ml of solution 1 with 823.5ml of solution 2

Chromomycin A_3

Dissolve 10mg CMA_3 in 1ml ethanol

Store at -20 ºC in 25µl aliquots

Add 125µl of McIlvaine's buffer (working solution) to each aliquot before using

Fixative

Methanol: acetic acid (3:1)

Dabco Anti-Fade Solution

Method

CMA_3 Staining:

1. Prepare smears, and allow air-drying.
2. Fix smears for 20 minutes at room temperature in methanol: acetic acid, and allow air-drying.
3. Stain slides for 20 minutes with 30µl CMA_3 in a dark chamber.
4. Rinse slides in McIlvaine's buffer.

Mounting:

5. Mount slides immediately with Dabco anti-fade solution.
6. Cover slides with coverslips; avoid the occurrence of air bubbles.
7. Store slides at room temperature overnight and evaluate the next morning.

Evaluation:

8. Slides are evaluated using a fluorescence microscope.
9. Find a field of spermatozoa under 10 x magnification (Phase Contrast).
10. Use immersion oil and change to the 100 x phase contrast objective.
11. Count one hundred spermatozoa and evaluate the sperm head for staining quality.
12. The percentage bright yellow stained spermatozoa are counted as CMA_3 positive cells. These spermatozoa are regards as cells with poor chromatin packaging quality. Poor chromatin packaging quality in the sperm head are indicated by:
    - Faintly yellow fluorescent staining;
    - Bright yellow fluorescent staining.
13. Dull yellow stained spermatozoa are counted as CMA_3 negative cells and contain good chromatin packaging. Good chromatin packaging quality in the sperm head are indicated by:
    - No fluorescent staining;
    - Fluorescence band at equatorial segment;
    - Fluorescent stain around periphery of head (stain did not permeate membrane).
14. The spermatozoa are classified as mature (good) versus immature (poor) DNA packaging (Esterhuizen *et al.,* 2000).

ADDENDUM IV

TUNEL ASSAY

Materials

Phosphate-Buffered Saline (PBS)

4% Formaldehyde

0.2% Triton X-100

DeadEnd™ Fluorometric TUNEL System

Method

1. Wash spermatozoa by centrifugation at 300 x g for 10 minutes in PBS.
2. Discard the supernatant and suspend the pellet.
3. Prepare smears by adding 10-50µl of sperm suspension onto Starfrost slides, and allow air-drying.
4. Fix smears for 25 minutes in 4% formaldehyde in PBS.
5. Wash slides for 5 minutes in PBS at room temperature.
6. Permeabilize cells for 5 minutes in 0.2% Triton X-100 in PBS.
7. Rinse slides twice for 5 minutes in fresh PBS at room temperature.
8. Remove excess liquid by tapping slides.
9. Add 100µl equilibration buffer onto slides and equilibrate for 5 minutes.
10. Prepare sufficient TdT-labelling solution.

Buffer component	Component volume per reaction		Number of slides		Total component volume
Equilibration buffer (in kit)	**45µl**	**X**	_____	**=**	_____**µl**
Nucleotide mix (in kit)	**5µl**	**X**	_____	**=**	_____**µl**
TdT-enzyme (in kit)	**1µl**	**X**	_____	**=**	_____**µl**

Total TdT-labelling solution volume = _____µl

11. Add 20µl TdT-labelling solution to slides; do not allow cells to dry out.
12. Cover slides with plastic coverslips.
13. Incubate slides for 1 hour at 37°C in a dark humidified chamber.
14. Remove coverslips and terminate the reaction by immersing slides in 2x SSC for 15 minutes at room temperature in the dark.
15. Wash slides twice for 5 minutes in deionized water at room temperature.
16. Analyse slides immediately.

Evaluation:

17. Slides are evaluated using a fluorescence microscope.
18. Find a field of spermatozoa under 10 x magnification (Phase Contrast).
19. Use immersion oil and change to the 100 x phase contrast objective.
20. Count one hundred spermatozoa and evaluate the sperm head for staining quality.
21. The Spermatozoa are classified according to intact or fragmented DNA.
    - Green nuclear fluorescence indicates fragmented sperm DNA.
    - No fluorescent staining is indicative of intact sperm DNA (Henkel *et al.,* 2004).

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