Studies on genetic aberrations as possible predictors of the outcome of assisted reproduction

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STUDIES ON GENETIC ABERRATIONS AS POSSIBLE PREDICTORS OF THE OUTCOME OF ASSISTED REPRODUCTION

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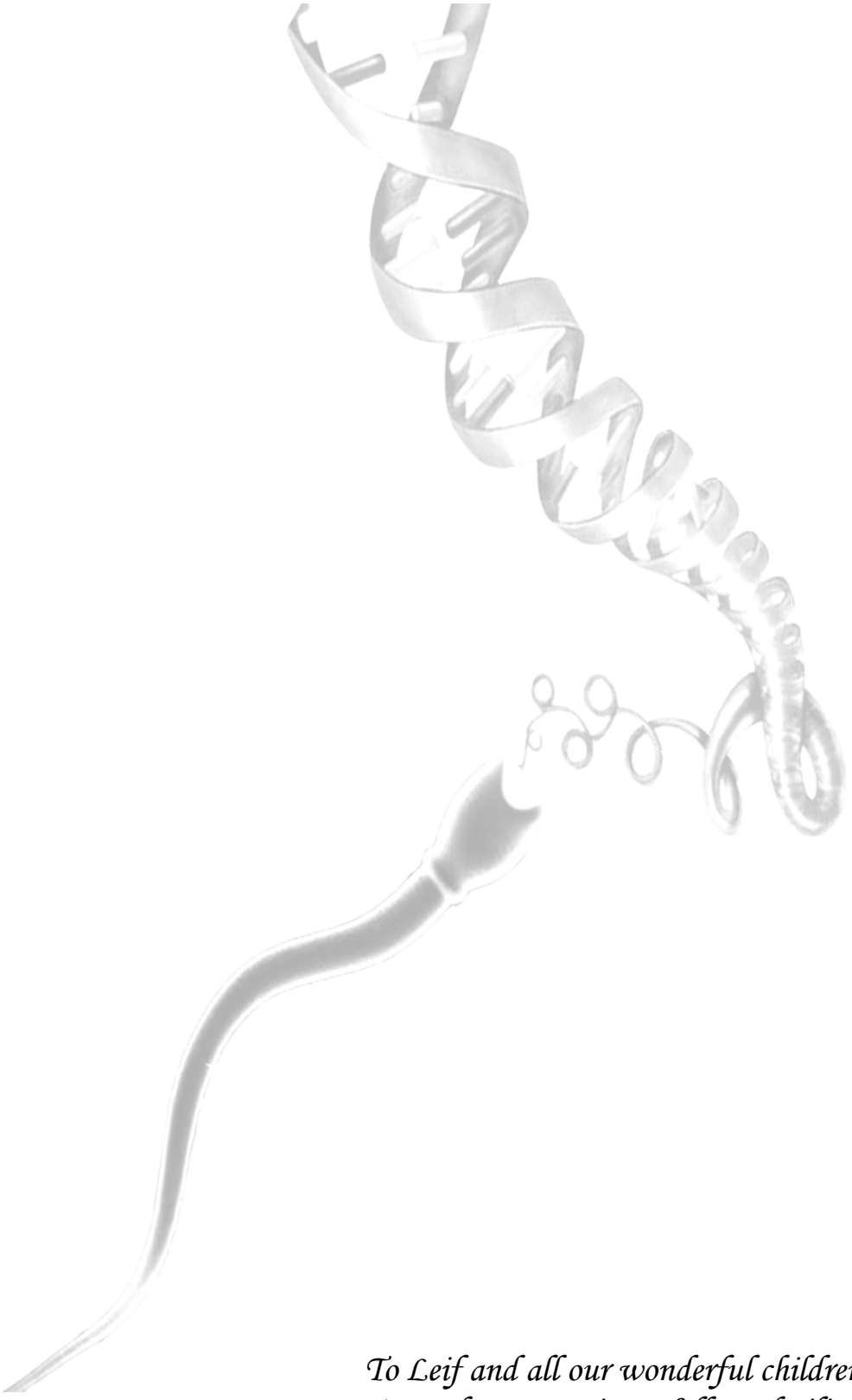

To Leif and all our wonderful children –
Ane, Ola, Lars, Linn, Helle and Silje

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ABBREVIATIONS

AO	acridine orange
ART	assisted reproductive techniques
BMI	body mass index
BP	biochemical pregnancy
BT	Bungum`s threshold
CASA	computer assisted sperm analyser
CBAVD	congenital bilateral absence of vas deferens
CI	confidence interval
CP	clinical pregnancy
CV	coefficient of variation
D	delivery
DFI	DNA fragmentation index
DGC	density gradient centrifugation
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSB	double strand breaks
EDTA	ethylene diamine tetracetate
ET	Evenson`s threshold
FSH	follicle stimulating hormone
hCG	human chorionic gonadotrophin
HCl	hydrogen chloride
HDS	high DNA stainability
ICSI	intra cytoplasmic sperm injection
IU	international units
IUI	intrauterine insemination
IUI-T	IUI-threshold
IVF	in vitro fertilisation
Kb	kilobase
KCl	potassium chloride
MAR	matrix attatch regions
OAT	oligoastheno-teratozoospermia
OR	odds ratio
OS	oxidative stress
PCI	protein C inhibitor
PCR	polymerase chain reaction
PN	pronuclei
ROS	reactive oxygen species
RNA	ribonucleic acid
SCSA	sperm chromatin structure assay
SD	standard deviation
SNP	single nucleotide polymorphism
SSB	single strand breaks
TUNEL	terminal deoxynucleotidyl transferase-mediated nick end labeling
WHO	world health organisation
ZP	zona pellucida

PREFACE

This thesis comprises two parts. The first part contains a review of the literature in the field. Subsequently the aims of the thesis are presented. Moreover, part one contains an overview of the materials and methods used for the studies, presentation of the results as well as a discussion of the findings of the five studies the thesis is based on. Finally, a conclusion and some future perspectives are drawn. The second part of the thesis comprises the published Papers (I-IV) and one submitted manuscript (V) on which the present thesis is based.

LIST OF ORIGINAL PAPERS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-V):

I **Bungum M,** Humaidan P, Spano M, Jepson K, Bungum L and Giwercman A. The predictive value of Sperm Chromatin Structure Assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Human Reproduction 2004,19:1401-1408.*

II **Bungum M,** Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J and Giwercman A. Sperm DNA integrity assessment in prediction of outcome of assisted reproduction. *Human Reproduction 2007,22:174-179.*

III **Bungum M**, Spano M, Humaidan P, Eleuteri P, Rescia M and Giwercman A. Sperm Chromatin Structure Assay (SCSA) parameters measured after density gradient centrifugation are not predictive for the outcome of ART. *Human Reproduction 2008,23:4-10.*

IV Erenpreiss J, **Bungum M**, Spano M, Elzanaty S, Orbidans J and Giwercman A. Intra-individual variation in Sperm Chromatin Structure Assay parameters in men from infertile couples: clinical implications. *Human Reproduction 2006,21:2061-2064.*

V **Bungum M,** Giwercman A, Bungum L, Humaidan P, Rastkhani H and Giwercman YL. Role of the Protein C Inhibitor (PCI) gene in IVF fertilisation failure. *Submitted.*

BACKGROUND

Infertility

One of the most common disorders in the western world is infertility, defined as inability to conceive after 12 months of regular intercourse in the absence of contraceptives. As many as 10-15% of couples have difficulties of conceiving, and seek medical care during their reproductive lifetime. Recent studies show that the number of infertile couples in the general population is growing (Feng 2003). Most patients are subfertile, rather than sterile (infertile), but the degree of subfertility is difficult to predict (Baker 2001). A fertile partner may compensate for a less fertile spouse and thus in most cases the term subfertility better covers the condition.

For a long time, female factors have been regarded as the primary causes of failure to conceive. However, in 20% of involuntarily childless couples, the predominant cause is male related, and in another 27%, anomalies in both partners contribute to childlessness (WHO 2000). Genital infections, endocrine disturbances and immunological factors have been regarded as the most common causes of male subfertility. However, genetic and other molecular causes have been identified as contributing explanatory factors to an increasing degree (Ferlin *et al.,* 2007b). In 60-75% of the male caused cases the aetiology of reduced semen quality remains unexplained and is referred to as idiopathic infertility (WHO 2000), why causal treatment is impossible (Skakkebaek *et al.,* 1994).

Traditionally, great care has been taken to obtain a diagnosis concerning the cause of female subfertility. More rarely a clinical evaluation of the man to find the underlying cause of the abnormal semen analysis is performed (Dohle 2007). Furthermore, the sperm parameters used in diagnosis are claimed to be poorly standardized, subjective (Auger *et al.,* 2000), and not powerful predictors of male fertility (Bonde *et al.,* 1998; Guzick *et al.,* 2001).

Today, many subfertile couples can be helped successfully by the use of assisted reproductive techniques (ART). In particular, the introduction of intracytoplasmatic sperm injection (ICSI) has given almost every involuntarily childless couple hopes of parenting. However, recent knowledge regarding male genetic causes to infertility has raised concern about the widespread use of ART and in particular ICSI to overcome male infertility. ICSI bypasses natural biological barriers that prevent against fertilisation with defective sperm. Although the new techniques have brought us further and led to a vast increase in our understanding of early reproductive physiology, the results of ART are still relatively low; baby-take-home rates of 20-30% having been held stable during the last two decades (Andersen *et al.,* 2005). One of the reasons for this is a lack of adequate methods to evaluate the fertility potential of a couple and also a lack of methods to identify the most effective type of ART treatment for a given couple.

Causes of male subfertility

Reduced male fertility can be the result of congenital and acquired urogenital abnormalities, infections of the genital tract, varicocele, endocrine disturbances, genetic or immunological factors (Figure 1). However, in 60-75% of the men, no causal factor behind the impaired semen parameters is found (idiopathic male subfertility) (WHO 2000). These men present with no previous history associated with fertility problems and have normal findings on physical examination and endocrine laboratory testing (WHO 2000). In some of these men, genetic causes can be found, but in most cases more likely genetic predisposition in combination with environmental compounds play a role in their hampered reproductive function (Skakkebaek *et al.,* 2001; Sharpe and Irvine 2004). Figure 1 summarizes the main aetiological causes of male subfertility.

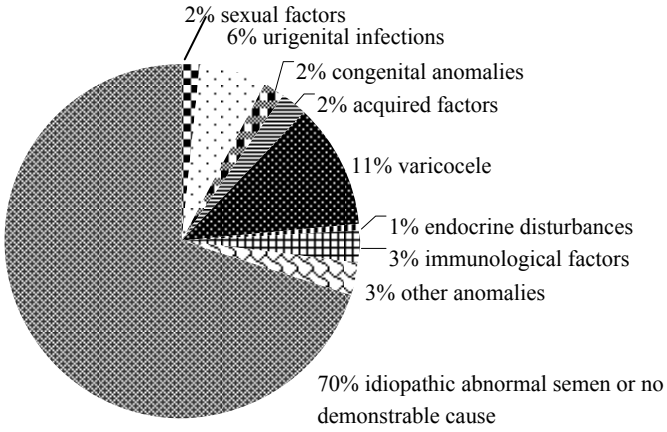

Figure 1. Aetiology and distribution of male infertility (From WHO, 2000).

Risks for couples undergoing IVF and ICSI are related to transmission of constitutional genetic abnormalities, genetic alterations present only in sperm or de-novo generated genetic disorders (Foresta *et al.,* 2002). Genetic abnormalities can be divided into categories as shown in Table 1.

Table 1. Genetic abnormalities that can be transmitted by sperm (modified from (Marchetti and Wyrobek 2005)).

Chromosomal aberrations	**DNA defects**
Aneuploidy • *Sex chromosomes* • *Autosomes* Structural aberrations • *Duplications/deletions* • *Rearrangements* Epigenetic changes • *Imprinting*	DNA lesions • *DNA adducts* • *Protamine adducts* • *Single and double DNA breaks* Sequence changes • *Gene mutations* • *Polymorphisms*

The prevalence of chromosomal abnormalities is higher in infertile men, this figure being inversely related to sperm count. Although sex chromosome abnormalities are predominant, also a wide range of abnormalities in the autosoms can be found (Ferlin *et al.,* 2007b). Chromosomal abnormalities can be divided into numerical (aneuploidy) and structural abnormalities (translocations). Klinefelter's syndrome (47, XXY) is the most common sex chromosome disorder occurring in 0.2 % of newborn boys (reviewed in (Smyth and Bremner 1998)). Most adult men with Klinefelter's syndrome are fertile, but the disorder is also associated with oligospermia (Mau-Holzmann 2005). Among infertile men, the prevalence of Klinefelter's syndrome is very high, up to 5% in severe oligozoospermia and 10% in azoospermia (Foresta *et al.,* 2005).

Also men with reciprocal balanced translocations often present with reduced fertility (Ferlin *et al.,* 2007b). Their offspring inherit unbalanced genetic material, receiving either too much or too little on the different chromosomes and the pregnancies often end with miscarriages (Ferlin *et al.,* 2007b).

Deletions of the non-recombining region of the Y-chromosome account for the infertility observed in about 15% of patients with azoospermia and 5–10% with severe oligozoospermia (Krausz and Degl'Innocenti 2006; Ferlin *et al.,* 2007a). Microdeletions have been found in four regions of the Y chromosome, AZFa-b-c-d, deletions in the AZFc region beeing considered as the most common (Vogt *et al.,* 1996; Kent-First *et al.,* 1999; Muslumanoglu *et al.,* 2005). Y-deletions can be transmitted to male offspring. However, most often this occurs by the use of ICSI, as men with very low sperm counts are less likely to father children spontaneously (Vogt *et al.,* 1996; Pryor *et al.,* 1997; Kent-First *et al.,* 1999; Mau Kai *et al.,* 2008).

The analysis of polymorphisms in genes involved in spermatogenesis represents one of the most growing areas of research in genetics of male infertility. Genetic variants are considered potential risk factors, which may contribute to the severity of spermatogenic failure and male infertility (Reviewed in (Ferlin *et al.,* 2007b)). Examples of such are mutations in the androgen receptor gene (Giwercman *et al.,* 2001), in the cystic fibrosis transmembrane conductance regulator gene (CFTR) (Foresta *et al.,* 2005) and in the KALIG-1 gene (Kallmann's syndrome) (Franco *et al.,* 1991). However, for several of these variants it was demonstrated that phenotypic effects of gene polymorphisms are modulated by other genetic factors or genetic background and environmental factors, providing an important example of a gene-environment interaction in phenotype development. Therefore, it is likely that polymorphisms only in association with a specific genetic background and/or with environmental factors can lead to spermatogenetic impairment or testicular dysfunction (Reviewed in (Giwercman *et al.,* 2007)).

Single base mutations in relation to human male infertility are only occasionally reported (reviewed in (Hiort and Holterhus 2003)). However, in animal studies mutations in several genes associated with or suggested to play a role in male infertility are identified (reviewed in Ferlin *et al.* 2007b), but so far often the role is unknown in humans. One of these genes studied in knock-out mice (Uhrin *et al.,* 2000) and suggested to play a role also in human male fertility is the Protein C inhibitor (PCI) gene (Espana *et al.,* 2007).

The Protein C inhibitor gene

The human PCI gene is located in a cluster with other serine protease inhibitors on chromosome 14q32.1 (Billingsley *et al.,* 1993). It is 11.5 kb long and comprises six exons (Meijers and Chung 1991) (Figure 2).

Protein C inhibitor is a serine protease inhibitor that modulates the activity of several blood-clotting factors such as activated protein c, factor Xa, thrombin, plasma kallekrein and prostate specific antigen (Espana *et al.,* 1989; Christensson

and Lilja 1994). PCI primarily inhibits the thrombin/thrombomodulin complex, where thrombin plays an anticoagulant role in blood (Pike *et al.,* 2005).

Some studies have suggested that PCI also could play a role in male fertility (reviewed in (Espana *et al.,* 2007)). PCI is expressed in different organs and tissues. It has also been shown to be present in the testis and the prostate and on the acrosomal cap of human sperm (Moore *et al.,* 1993). In fact, the highest PCI concentration has been measured in seminal plasma (Laurell *et al.,* 1992) and in seminal vesicle secretions (Espana *et al.,* 1991).

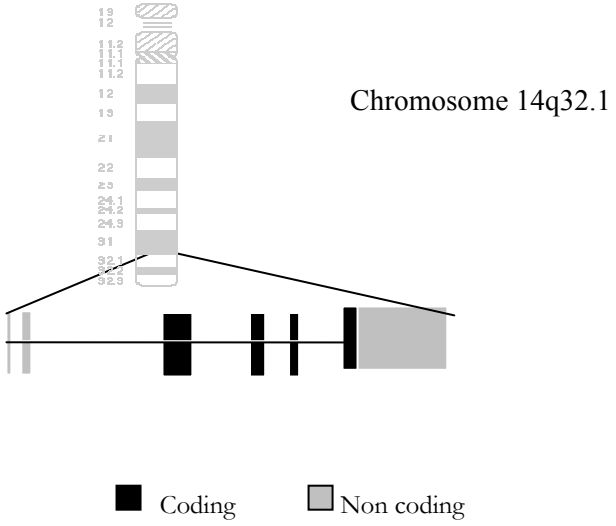

Figure 2. The PCI gene, located in a cluster with other serine protease inhibitors on chromosome 14q32.1 (Billingsley *et al.,* 1993).

Studies have demonstrated that PCI acts as a rapid inhibitor of acrosin, a serine protease stored in the acrosome of sperm (Hermans *et al.,* 1994; Elisen *et al.,* 1998). Recently, an Austrian group reported that in male mice, the presence of PCI is an absolute requirement for reproduction (Uhrin *et al.,* 2000). Male PCI knockout mice produced normal amounts of sperm, but these were morphologically abnormal and unable to penetrate the oocytes. The sequence and the amino acid sequence deduced from the mouse PCI gene are highly homologous with the human PCI gene (Zechmeister-Machhart *et al.,* 1997; Uhrin *et al.,* 2000).

Despite this fact, the role of PCI gene in human fertility is unknown. At the time when the present study was initiated, only one human study on the role of the PCI gene in fertility was published (Gianotten *et al.,* 2004). Gianotten and co-workers studied a group of infertile men, diagnosed with idiopathic azoospermia or teratozoospermia. Although Gianotten's group found several new mutations in the PCI gene, they were not able to link them to male infertility. They concluded that mutations in the PCI gene are not a common cause of reduced semen parameters. Nevertheless, PCI could play a role in male infertility. Seen in the light of the findings in PCI knocked-out mice (Uhrin *et al.,* 2000), Gianotten's study population was not optimal, as it might include a heterogeneous group of men with subnormal sperm parameters and not only those with specific disability of the sperm to penetrate zona pellucida (ZP) of the oocyte, as was the finding in the animal studies. Thus, at the time of initiating the work behind this thesis the association between PCI gene variants and infertility was still not fully elucidated.

Sperm DNA and chromatin structure

Generally overlooked in the diagnosis and treatment of male infertility is the fact that sperm carry DNA (deoxyribonucleic acid) and that the DNA can be of a different quality. The nuclear DNA, commonly called the genome, is located in the head of the sperm. The second DNA type is called the mitochondrial DNA and is responsible for delivering the sperm to the egg by providing energy for cellular acceleration. Both types of DNA work toward the common goal of fertilisation, but each is susceptible to a huge number of factors that could derail the fertilisation process (Lewis and Aitken 2005). This thesis will only discuss nuclear DNA.

Human sperm chromatin differs from chromatin in both human somatic cells and from sperm cells in other mammals, in structure as well as composition. In humans up to 15% of the sperm DNA is packaged by histones in sequence-specific areas (Gatewood *et al.,* 1987). These histone-bound DNA sequences are less tightly compacted and suggested to be involved in fertilisation and early embryonic development (Gatewood *et al.,* 1987; Gardiner-Garden *et al.,* 1998). During the final stage of spermatogenesis (spermiogenesis) where the round spermatids mature into elongated, motile sperm, somatic-type histones are firstly replaced with testis-specific histones, followed by transition proteins and, finally, by the sperm-specific proteins, protamines (Poccia 1986). DNA and protamines are further organized into unique supercoiled doughnuts, called toroids, each containing 50-60 kb of DNA (Ward and Coffey 1991), fixed at the nuclear matrix (Fuentes-Mascorro *et al.,* 2000) (Figure 3). Each chromosome represents a garland of toroids, and all 23 chromosomes in the sperm are clustered by centromeres into a compact chromocenter positioned well inside the nucleus (Ward 1993).

In contrast to most other mammals, in which sperm DNA is associated with only one protamine (P1), human spermatozoa have two types of protamines (P1 and P2) (Oliva 2006; Carrell *et al.,* 2007). P2 has fewer thiol groups for disulphide bonding, which makes human sperm chromatin less stable than the chromatin of other mammals (Jager 1990; Jager *et al.,* 1990). Both an altered P1/P2 ratio and the absence of P2 are associated with male fertility problems in humans (Balhorn *et al.,* 1988; de Yebra *et al.,* 1993; Bench *et al.,* 1998; Carrell and Liu 2001). The condensed and highly organized nature of sperm chromatin protects the paternal genome during the transport through the reproductive tracts (Ward and Zalensky 1996; Solov'eva *et al.,* 2004).

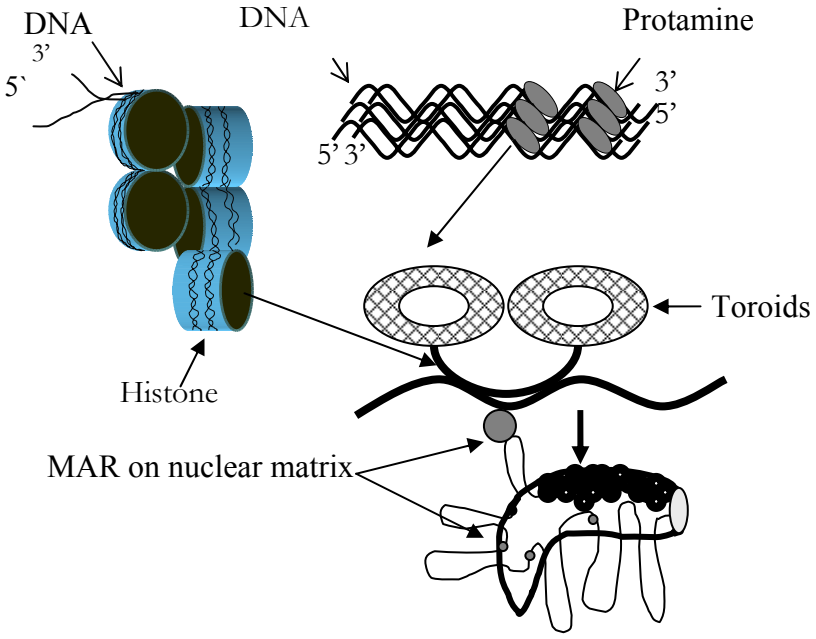

Figure 3. Human sperm chromatin structure, schematic presentation. Approximately 85% of the DNA is protamine-bound and 15% is histone-bound. The DNA near the matrix attatch regions (MAR) is associated with histones, not protamines (modified from Ward and Coffey, 1991).

Sperm DNA damage

It is evident that some of the ejaculated spermatozoa possess a variety of abnormalities at the nuclear, cytoskeletal, and organelle levels and that these anomalies can have an impact on fertility (Evenson *et al.,* 1980; Hewitson 1999; Huszar 1999). There is now clear evidence that infertile men possess substantially more sperm DNA damage than fertile men (Evenson *et al.,* 1980; Evenson *et al.,* 1999; Gandini *et al.,* 2000; Host *et al.,* 2000b; Irvine *et al.,* 2000; Larson *et al.,* 2000; Spano *et al.,* 2000; Carrell and Liu 2001; Hammadeh *et al.,* 2001; Zini *et al.,* 2001a; Sakkas *et al.,* 2002; Saleh *et al.,* 2002b; Zini *et al.,* 2002; Erenpreisa *et al.,* 2003; Muratori *et al.,* 2003; Saleh *et al.,* 2003a). This is clinically relevant in cases where infertile men will be treated with ART.

The amount and type of sperm damage is a direct consequence of the special biology of spermatogenesis as well as the first stages of zygote and embryo development. Human sperm chromatin is often poorly compacted (Sakkas *et al.,* 1999a) and is therefore susceptible to natural or induced DNA damage (Irvine *et al.,* 2000). Whilst the mature sperm itself has no DNA repair capacity (Sega *et al.,* 1978) oocytes and early embryos have been shown to repair sperm DNA damage (Matsuda and Tobari 1988), but only up to a certain extent (Ahmadi and Ng 1999b). Furthermore, insufficient or aberrant sperm DNA repair by the oocyte is hypothesized to create mutations in the genome of the zygote, which may lead to implantation failure, early miscarriages or in the worst cases diseases in the offspring (Agarwal and Said 2003; Aitken and Baker 2004; Liu *et al.,* 2004).

In addition to the above mentioned aberrations also larger damage may occur, resulting in chromatin breaks and sperm DNA fragmentation as a consequence of poor chromatin packaging, apoptosis or damage induced by oxidative stress (OS) (reviewed in (Erenpreiss *et al.,* 2006; Aitken and De Iulius 2007)).

Causes of sperm DNA damage

Spermatogenesis is a complex process of male germ cell proliferation and maturation from diploid spermatogonia through meiosis to mature haploid spermatozoa (de Kretser *et al.,* 1998) where damage of sperm DNA or its chromatin structure can occur at any step (reviewed in (Erenpreiss *et al*., 2006). Various hypotheses have been proposed as to the molecular mechanism of sperm DNA damage. First of all the chromatin can be loosely or abnormally packaged due to underprotamination, which results in endogenous nicks in the DNA (DNA strand breaks or DNA fragmentation) (Manicardi *et al.,* 1995; Sakkas *et al.,* 1999a). This can happen by natural or induced processes. Natural processes involve poor chromatin remodelling as a consequence of inadequate protamination and the chromatin remains histone rich, apoptosis and that DNA breaks induced in

meiosis and in spermatids (Laberge and Boissonneault 2005) are not repaired (reviewed in (Aitken and De Iulius 2007). Induced problems involve for instance cancer treatment, infections, fever, air pollution, cigarette smoking, obesity, advanced age and preparation for ART (reviewed in (Erenpreiss *et al.,* 2006; Aitken and De Iulius 2007)). A brief schematic presentation of the factors contributing to DNA damage is shown in Figure 4.

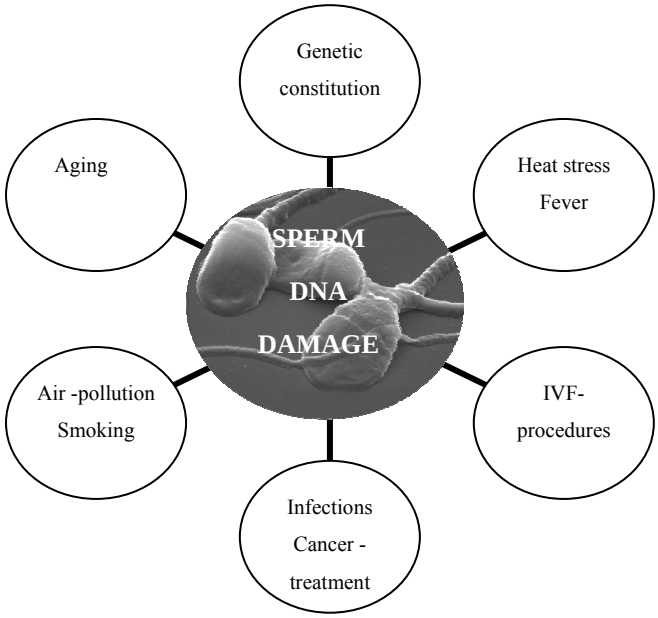

Figure 4. Proposed causes of sperm DNA fragmentation.

Despite the fact that the origin and the mechanisms responsible for sperm DNA breaks and fragmentation are not fully understood, three potential sources at the molecular level are suggested (Sakkas *et al.,* 1999b; Agarwal and Said 2003); (1) alterations in sperm chromatin packaging; (2) abortive apoptosis; and (3) oxidative stress (OS). Most likely, these factors are interrelating. For instance, a defective checkpoint in regard to crossing-over during spermatogenesis or deficiencies in the protamination process is likely to make sperm more vulnerable to later oxidative stress.

Meiotic crossing-over during spermatogenesis is associated with the programmed introduction of DNA double strand breaks (DSBs), expected to be ligated until the end of meiosis I (Bannister and Schimenti 2004).

In animal (McPherson and Longo 1993; Sakkas *et al.,* 1995) as well as in human studies (Marcon and Boissonneault 2004) stage-specific introduction of transient DNA strand breaks during spermiogenesis have been described. DNA breaks induced by DNA Topoisomerase II, have been found in round as well as elongating spermatids. DNA breaks are necessary for transient relief of torsional stress, favouring casting off of the nucleosome histone cores, and aiding the histone replacement with protamines during the final maturation form round to elongated spermatozoa (McPherson and Longo 1993; Marcon and Boissonneault 2004; Laberge and Boissonneault 2005). However, ligation of DNA breaks is also necessary; not only to preserve the integrity of the primary DNA structure, but also to reassembling the important unit of genome expression - the DNA loop domain. If these physiological, normal temporary breaks are not repaired, DNA fragmentation in ejaculated spermatozoa or genetic mutations may occur (Aitken 1999; Aitken *et al.,* 2004).

The second suggested aetiology of DNA damage is the fact that the breaks/fragmentation can arise through an abortive apoptotic pathway. Apoptosis of testicular germ cells occurs normally throughout life, controlling overproliferation (Billig *et al.,* 1995; Rodriguez *et al.,* 1997). It has been suggested that an early apoptotic pathway, initiated in spermatogonia and spermatocytes, is mediated by the Fas protein, a type I membrane protein belonging to the tumour necrosis factor–nerve growth factor receptor family (Suda *et al.,* 1993). Sertoli cells in the testis express Fas ligand, which by binding to Fas leads to cell death through apoptosis (Suda *et al.,* 1993). Sakkas and co-workers (Sakkas *et al.,* 1999a) demonstrated that men with abnormal sperm parameters have an increased number of spermatozoa bearing Fas compared to men with normal semen parameters. However, However, Sakkas himself and others found no correlations between DNA damage and Fas expression (Muratori *et al.,* 2000; Sakkas *et al.,* 2002).

Finally, the mechanism that probably most evidence exists for is oxidative stress (OS). OS is caused by an imbalance between the antioxidant ability in seminal plasma and the production of reactive oxygen species (ROS). While oxygen is essential to all aerobic life, it becomes toxic when administered in too high concentrations, as it may produce ROS that will have beneficial as well as detrimental effects on the cells, depending on the nature and concentration (reviewed in (Aitken and Baker 2004)). Spermatozoa are extremely vulnerable to OS. The sperm cell membrane, being rich on unsaturated fatty acids, is easily attacked by ROS with further detrimental effects on nuclear membranes as well as

on sperm DNA (Aitken and Krausz 2001). Furthermore, sperm lack antioxidants and DNA repair systems (Aitken *et al.,* 2003), and are therefore completely dependant on the repair capacity of the oocyte and the early embryo.

Leukocytes and abnormal spermatozoa in the semen are among the main sources of ROS in semen (Aitken *et al.,* 1992; Alvarez *et al.,* 2002; Saleh *et al.,* 2002a) seen more often in semen from men with leukocytospermia than in healthy donors (Alvarez *et al.,* 2002; Saleh *et al.,* 2002a). DNA damaged sperm caused by increased scrotal temperature due to illness with fever (Evenson *et al.,* 1991; Evenson and Jost 2000; Sergerie *et al.,* 2007) or varicocele (Saleh *et al.,* 2003b) is also reported. Studies on patients with testicular cancer have shown that sperm DNA might be damaged even before irradiation and chemotherapy (Evenson *et al.,* 1984; Fossa *et al.,* 1997; Kobayashi *et al.,* 2001). However, cancer therapy has been shown to further contribute to increased DNA damage (Kobayashi *et al.,* 2001; Stahl *et al.,* 2004; 2006). Moreover, older men are reported to have sperm with more DNA fragmentation than younger men (Spano *et al.,* 1998; Singh *et al.,* 2003; Moskovtsev *et al.,* 2006; Wyrobek *et al.,* 2006; Plastira *et al.,* 2007). An aberrant repair of sperm DNA damage by the oocyte has been proposed as as age-related causative mechanism of DNA damage (Aitken 1999; Aitken and Baker 2004). As the trend today is increased parental age at childbirth, problems with sperm DNA damage are likely to increase.

Other proposed sources of ROS come from outside the sperm's immediate environment, usually from outside of the host's body. They include xenobiotic agents such as organophosphorous pesticides (Sanchez-Pena *et al.,* 2004) and other types of air pollution (Rubes *et al.,* 1998; Selevan *et al.,* 2000; Evenson and Wixon 2005; Jafarabadi 2007). These agents possess estrogenic properties that are capable of inducing ROS production in male germ cells (Sanchez-Pena *et al.,* 2004; Baker and Aitken 2005; Spano *et al.,* 2005b; Bennetts *et al.,* 2008). From animal studies deleterious effects of different toxicants are known to have a negative impact on sperm chromatin (Evenson *et al.,* 1986; Evenson *et al.,* 1987; Evenson *et al.,* 1993a; Evenson and Jost 1993; Evenson *et al.,* 1993b; Evenson *et al.,* 1993c; Spano *et al.,* 1996; Traina *et al.,* 2003). Moreover, smokers have an increased level of oxidative damage in their sperm DNA compared to non-smokers (Fraga *et al.,* 1996). Thus, several studies have reported a negative effect of cigarette smoking on sperm DNA (Robbins *et al.,* 1997; Sun *et al.,* 1997; Rubes *et al.,* 1998; Potts *et al.,* 1999; Saleh *et al.,* 2002b; Sepaniak *et al.,* 2006), as smoking has mutagenic properties, a fact associated with an overall reduction in the traditional semen parameters (Kunzle *et al.,* 2003).

Another potential source of OS in sperm is the procedures performed during ART. In a vast majority of cases, spermatozoa used for ART are prepared by density gradient centrifugation (DGC) or by a swim-up preparation in order to favour the

isolation of motile and morphologically normal spermatozoa (Sakkas *et al.,* 2000; Zini *et al.,* 2000; Tomlinson *et al.,* 2001; Morrell *et al.,* 2004; Allamaneni *et al.,* 2005). The media used for DGC consist of a colloidal silica suspension in a culture medium. The system separates normal sperm from lymphocytes, leukocytes, epithelial cells, abnormal or immature sperm, cell debris, bacteria and seminal fluid. Although the potentially compromised spermatozoa can be further damaged during centrifugations (Aitken and Clarkson 1988), these sperm preparation methods are still a standard in sperm preparation for ART. Furthermore, exposure to other potential hazards in the laboratory environment such as suboptimal culture media (Sikka 2004) or culture conditions (Dalzell *et al.,* 2003), cryopreservation (Chatterjee and Gagnon 2001) and light (Agarwal *et al.,* 2006) have been shown to increase the production of ROS, likely, with a negative impact on the sperm DNA as a concequence.

Possible impact of sperm DNA damage on fertility

During recent years several tests have been developed to assess sperm chromatin integrity. When this thesis was planned, the sperm chromatin structure assay (SCSA) (Evenson *et al.,* 1980; Evenson and Jost 2000; Spano *et al.,* 2000), designed to measure sperm DNA integrity as a complementary diagnostic laboratory analysis had been introduced. So far, SCSA had mainly been used in epidemiological studies of male fertility (Larsen *et al.,* 1998; Evenson *et al.,* 1999; Kolstad *et al.,* 1999; Spano *et al.,* 2000; Bonde *et al.,* 2002; Bonde *et al.,* 2003; Rignell-Hydbom *et al.,* 2005; Rubes *et al.,* 2005) and in toxicological studies of rodents (Evenson *et al.,* 1989; Evenson *et al.,* 1993a; Evenson and Jost 1993; Evenson *et al.,* 1993b; Spano *et al.,* 1996; Traina *et al.,* 2003).

The SCSA is a flowcytometric test that measures the susceptibility of sperm DNA to acid-induced DNA denaturation in situ, followed by staining with acridine orange (Evenson *et al.,* 1980; Spano *et al.,* 2000; Evenson *et al.,* 2002). DNA denaturation is determined by measuring the shift from green fluorescence (double-stranded, native DNA) to red fluorescence (single-stranded, denatured DNA) in a flow-cytometer, followed by further analysis by a dedicated SCSA-software. The extent of DNA denaturation is expressed in terms of the DNA fragmentation index (DFI) (Evenson *et al.,* 2002). Another SCSA parameter is the fraction of high DNA stainable (HDS) cells thought to represent immature spermatozoa with an incomplete protamination (Evenson *et al.,* 2002).

In addition to the SCSA, the Comet assay (single cell gel electrophoresis) (Morris *et al.,* 2002) and the TUNEL (terminal deoxynucleotidyl transferase-mediated dUDP nick end labelling) assay (Gorczyca *et al.,* 1993) are frequently used. Comet, TUNEL and SCSA all label single or double stranded DNA breaks. Good

correlations between the tests have been reported (Gorczyca *et al.,* 1993; Aravindan *et al.,* 1997; Erenpreiss *et al.,* 2004).

At the beginning of my studies, there were already several reports regarding higher fraction of sperm with DNA defects in infertile men than in fertile controls (Evenson *et al.,* 1980; Evenson *et al.,* 1999; Gandini *et al.,* 2000; Host *et al.,* 2000b; Irvine *et al.,* 2000; Larson *et al.,* 2000; Spano *et al.,* 2000; Carrell and Liu 2001; Hammadeh *et al.,* 2001; Zini *et al.,* 2001a; Sakkas *et al.,* 2002; Saleh *et al.,* 2002b; Zini *et al.,* 2002; Erenpreisa *et al.,* 2003; Muratori *et al.,* 2003; Saleh *et al.,* 2003a). In the Georgetown study, including 200 couples trying to conceive naturally, Evenson and co-workers found that the odds ratio was 6.5 times higher for a successful pregnancy if the DFI was <30% (Evenson *et al.,* 1999). In another time to pregnancy (TTP) study, the so-called Danish First-Pregnancy Planner study, Spano *et al.* (Spano *et al.,* 2000) reported a decreased fecundity rate with increasing number of DNA breaks in the ejaculate. Evenson *et al.* and Spano *et al.* suggested a cut-off value for DFI in regard to subfertility to be set at 30-40% (Table 2).

Furthermore, sperm DNA defects were also suspected to have a possible negative impact on the outcome of ART (Lopes *et al.,* 1998b; Larson *et al.,* 2000) and it was questioned whether ART was able to compensate for poor DNA quality (Twigg *et al.,* 1998; Evenson *et al.,* 1999; Larson *et al.,* 2000; Larson-Cook *et al.,* 2003). In a small study consisting of 19 couples, the chance of obtaining a pregnancy by IUI was extremely low when the proportion of sperm cells with DNA damage exceeded 30 % by means of SCSA (Saleh *et al.,* 2003a). Also using the TUNEL assay, it was demonstrated that in semen samples with >12 % sperm DNA fragmentation, no pregnancy occurred (Duran *et al.,* 2002) (Table 2).

Table 2. Influence of sperm DNA damage on pregnancy rates for spontaneous pregnancy and IUI treatment.

Author/Year of publication	Patients	In vivo method	Pregnancy rates	Test used	DFI-threshold suggested
Evenson, 1999	165+115	spontaneous pregnancy	↓	SCSA	30%
Spano, 2000	215	spontaneous pregnancy	↓	SCSA	40%
Duran, 2002	154	IUI	↓	TUNEL	12%
Saleh, 2003	19	IUI	↓	SCSA	30%

Also for the outcome of in vitro fertility, some smaller studies had indicated that DFI may be used as a prognostic factor (Larson *et al.,* 2000, Saleh *et al.,* 2003a, Larson-Cook *et al.,* 2003, Tomlinson *et al.,* 2001, Henkel *et al.,* 2003). A pilot study including 24 men (Larson *et al.,* 2000) demonstrated that when DFI exceeded 27%, no pregnancy was obtained. Later, the same authors (Larson-Cook *et al.,* 2003) confirmed the findings in 89 couples undergoing IVF and ICSI. On the other hand, HDS did not predict ART pregnancy. Saleh and co-workers (Saleh *et al.,* 2003a) studied 10 couples undergoing IVF and four couples undergoing ICSI and found that DFI, but not HDS was negatively correlated to pregnancy. No pregnancy occurred when DFI was above 28%. The largest ART study published at that time was a study of Henkel et al. (Henkel *et al.,* 2003) including in total 208 IVF and 54 ICSI cycles. Henkel et al. found no correlation between sperm DNA fragmentation as measured by the TUNEL assay and pregnancy. On the other hand, Tomlinson *et al.* (Tomlinson *et al.,* 2001) found significantly higher amount of DNA damage in the group of patients who became pregnant during IVF treatment compared to those who failed to be pregnant. Based on their own results, where no pregnancy occurred when DFI was above 27%, Evensons group suggested a DFI threshold value of 27% in regard to ART subfertility (Larson *et al.,* 2000; Larson-Cook *et al.,* 2003). However, the suggestion was based on relatively small materials, reported by one author. Only larger data sets could provide clearer indications for whether threshold levels for DFI and HDS could be defined.

A correlation between sperm DNA damage and fertilisation rates and embryo development has been suggested. While some authors reported associations between increased DNA fragmentation and fertilisation rates after IVF and ICSI (Sun *et al.,* 1997; Morris *et al.,* 2002; Carrell *et al.,* 2003; Saleh *et al.,* 2003a), others did not see any impact of DNA fragmentation on fertilisation rates (Larson *et al.,* 2000; Morris *et al.,* 2002; Henkel *et al.,* 2003; Larson-Cook *et al.,* 2003). Tesarik *et al.* (Tesarik *et al.,* 2002) hypothesized that the paternal genome could play a role in early embryonic development, as early as in the first cell cycle, when sperm DNA was fragmented. However, recently this group found sperm DNA fragmentation only to be related to late paternal effect (Tesarik *et al.,* 2004). This could perhaps explain the diverging reports regarding correlations between sperm DNA fragmentation and embryo development after IVF and ICSI. While some authors reported decreased cleavage and embryo development with increasing DNA fragmentation (Morris *et al.,* 2002; Tomsu *et al.,* 2002), others did not find any associations (Lopes *et al.,* 1998a; Larson *et al.,* 2000; Tomlinson *et al.,* 2001; Benchaib *et al.,* 2003; Larson-Cook *et al.,* 2003). However, these studies were all based on relatively few individuals (Table 3).

Historically, recurrent pregnancy loss has been attributed to either genetic, structural, infective, endocrine or unexplained causes (reviewed in (Rai and Regan

2006)). An increased rate of sperm chromosome abnormalities has been reported in patients with recurrent miscarriage (Giorlandino *et al.,* 1998), but only 7% of fetal trisomies have been shown to arise from paternal meiotic errors (Robinson *et al.,* 1999). When my work started, sperm DNA fragmentation was also thought to play a role in unexplained recurrent pregnancy loss (Evenson *et al.,* 1999; Carrell *et al.,* 2003). In a non-ART material, using the SCSA, Evenson *et al.* (1999) demonstrated that the miscarriage rate was higher in fertile couples in which the spermatozoa of the partner had poor chromatin quality compared to those with low rates of DNA fragmentation. Thirtynine percentage of the miscarriages was predicted from the SCSA data. Also Carrell *et al.* (2003) reported that recurrent pregnancy loss was associated with higher levels of DNA damage. They evaluated the degree of sperm DNA fragmentation using the TUNEL assay on sperm from 24 couples with unexplained recurrent pregnancy loss compared to sperm from two control groups: donors of known fertility and unscreened men from the general population. The proportion of sperm with DNA fragmentation was increased in the group with recurrent pregnancy loss compared to both control groups. However, also these studies included a limited number of cases and thus, the issue is needed to be addressed in larger additional studies.

Although in a vast majority of cases, sperm used for ART are prepared by density gradient centrifugation (DGC) in order to favour the isolation of motile and morphologically normal spermatozoa, almost all ART-sperm DNA integrity studies so far (Larson *et al.,* 2000; Larson-Cook *et al.,* 2003; Saleh *et al.,* 2003a), had been performed on native semen. Several studies had shown that, even though various levels of efficiency were reported, both sperm separation methods were quite effective in sorting out spermatozoa with damaged DNA and poorly condensed chromatin as evaluated by the SCSA (Larson *et al.,* 1999; Spano *et al.,* 1999; Larson *et al.,* 2000; Zini *et al.,* 2000), the TUNEL assay (Morrell *et al.,* 2004) and the Comet assay (Donnelly *et al.,* 2000). At the time when this work was initiated, it was unclear whether SCSA parameters of semen samples prepared by density gradient centrifugation were of predictive value for the outcome of IVF or not. Several previous authors had recommended the necessity of studies aiming at clarifying whether processed semen could have a predictive value in ART (Tomlinson *et al.,* 2001; Spano *et al.,* 2005a).

Table 3 shows the information regarding influence of sperm DNA damage on fertilisation rates, embryo quality and pregnancy rates during IVF and ICSI available at the time where the current work was initiated.

Table 3. Influence of sperm DNA damage on fertilisation rates, embryo quality and pregnancy rates during IVF and ICSI.

Author/ Year of publication	IVF, n	ICSI, n	Fertilisation rates	Embryo quality	Pregnancy rates	Test used
Lopes, 1998	0	150	↓	No	NA	TUNEL
Host, 2000	50	61	↓	NA	NA	TUNEL
Tomlinson, 2001	140	0	No	No	↓	TUNEL
Tomsu, 2002	40	0	No	↓	↓	Comet
Morris, 2002	20	40	No	↓	NA	Comet
Benchaib, 2003	50	54	↓	No	↓	TUNEL
Larson-Cook, 2003	55	34	No	No	↓	SCSA
Larson, 2000	24 IVF/ ICSI	NA	No	No	↓	SCSA
Saleh, 2003	10	4	↓	↓	↓	SCSA
Henkel, 2003	208	54	No	No	No	TUNEL

Some studies had reported that DFI was a semen parameter with a lower variability than the traditional semen parameters (Neuwinger *et al.,* 1990; Cooper *et al.,* 1992; Keel 2006), exhibiting a coefficient of variation (CV) for intra-individual variation of around 10-20% (Evenson *et al.,* 1991; Zini *et al.,* 2001b; De Jonge *et al.,* 2004). In contrast, for the traditional sperm parameters, a CV for intra-individual variation as high as 54 % had been reported (Keel 2006). The studies reporting such a low CV for DFI, however, consisted of very few men. In the study of Zini *et al.* (2001b), the men provided two semen samples, 2 to 6 weeks apart and in the study by Evenson *et al.* (1991), 45 men delivered monthly semen samples during a period of 8 months. De Jonge *et al.* (2004) studied variation of DNA fragmentation

in relation to days of sexual abstinence in 11 men and found only a short (24-hour) abstinence period to have a negative influence on sperm chromatin quality.

All in all, findings from these smaller studies indicated that sperm chromatin integrity could possibly have the potential of being a measurable predictor of fertility in natural fertility as well as in ART. However, the possible clinical applications for sperm chromatin integrity testing in a clinical set up, had not yet been defined.

Diagnosis and treatment of male subfertility

In most clinics, the diagnosis male subfertility is based solely on the presence of abnormal semen. WHO has set criteria for normality in regard to the conventional sperm parameters: semen volume, sperm concentration, motility and morphology (WHO 1999) as shown in Table 4. In addition, other parameters like viscosity, pH and biochemistry of the seminal plasma are often examined.

Table 4. Reference values of semen parameters (from the WHO manual, 1999)

Parameters	**Reference values**
Semen volume	$\geq$ 2 ml
Sperm concentratrion	$\geq$ 20x 10^6/mL
Total sperm count	$\geq$ 40x 10^6/ejaculate
Motility	$\geq$ 25% rapid proggressive or
	$\geq$ 50% total proggressive motility
Morphology	Variable thresholds

The poor power of semen analysis has been pointed out by several authors (Bonde *et al.,* 1998; Giwercman *et al.,* 1999; Auger *et al.,* 2001; Guzick *et al.,* 2001; Nallella *et al.,* 2006; Swan 2006). One of the reasons for the lack of power of the analysis is the inherent heterogeneity of human semen. Concentration, motility and morphology vary significantly between individuals, seasons, countries and regions and even between consecutive samples from one individual (Chia *et al.,* 1998; WHO 1999; Auger *et al.,* 2000; Jorgensen *et al.,* 2001; Chen *et al.,* 2003; Jorgensen *et al.,* 2006). As semen analysis is mainly performed by manually light microscopy of 1-200 spermatozoa the analysis implies a high level of subjectivity. The value of the conventional semen parameter measurements is, therefore, also

limited due to intra- and interlaboratory variation (Neuwinger *et al.,* 1990; Cooper *et al.,* 1992). Moreover, an extensive overlap in sperm concentration, motility and morphology between fertile and infertile men are reported (Bonde *et al.,* 1998; Guzick *et al.,* 2001) (Table 5).

Table 5. Fertile, indeterminate, and subfertile ranges for sperm measurements (from Guzick *et al.* 2001)

	Fertile range	**Indeterminate range**	**Subfertile range**
Concentration, $x10^6$/ml	>63	13.5-48	<13.5
Sperm motility, %	>63	32-63	<32
Sperm morphology, %	>12	9-12	<9

Furthermore, several other laboratory tests of sperm function have been developed; antisperm antibody test, vital staining, biochemical analysis of semen, hypoosmotic swelling test, sperm penetration assay, hemizona assay, creatin-kinase, reactive oxygen species (ROS) tests and computer-assisted sperm analysis (CASA), to mention the most commonly used (Reviewed in (Aitken 2006)). However, the predictive and clinical value of these tests has also been questioned (Muller 2000), and in the WHO manual, none of them are directly recommended; just included as possible supplementary tests to the conventional sperm analysis (WHO 1999).

Although the origin and the mechanisms responsible for sperm DNA damage are not yet fully clarified, it has been proposed that sperm DNA integrity could be a possible fertility predictor to be used as an alternative or as a supplement to the traditional sperm parameters (Evenson *et al.,* 2000; Larson *et al.,* 2000). As already reviewed, in 2002, when the current work was initiated, there was some evidence that sperm DNA integrity could be an indicator of male fertility potential (Evenson *et al.,* 1999; Evenson and Jost 2000; Evenson *et al.,* 2002; Saleh *et al.,* 2002b; Saleh *et al.,* 2003b) and thereby possibly be an alternative or a supplement to the traditional analysis. However, regarding assisted reproduction, data was very limited and therefore, at that time, very few ART-programmes had implemented sperm chromatin integrity testing. A majority of the infertile couples were and are still referred directly to ART, without any other causal investigation than a standard semen analysis (Lewis 2007).

ART is defined as all reproductive technologies that involve handling of gametes outside the body, either sperm alone as in intrauterine insemination (IUI), or both eggs and sperm as in in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) (Edwards and Brody 1995). The very first documented use of ART was in 1783, when Spallanzani delivered pups from an artificially inseminated bitch. Close to 200 years later, in 1978, a British group reported the birth of Louise Brown, the worlds first IVF-baby (Steptoe and Edwards 1978). Now, ART is applied worldwide and it is estimated that more than three million babies have been born as a result of ART (Zegers-Hochschild *et al.,* 2006).

While the least invasive form of ART, i.e. IUI, where prepared semen is inseminated in the women's uterus, is used in ovulatory dysfunction, unexplained subfertility and milder forms of male subfertility, IVF is primarily used in female subfertility or as a second choice in unexplained causes (Edwards and Brody 1995). Originally, ICSI was developed to be used in severe male infertility cases, however, it is now in many clinics, the main method used for all indications (Jain and Gupta 2007; Andersen *et al.,* 2008). In IVF, oocytes are fertilised by sperm *in vitro*. Two to five days later the fertilised and cleaved oocyte (embryo) is transferred to the patient's uterus. For ICSI the same treatment principles are followed, however, here one single spermatozoon is injected directly into the cytoplasm of the oocyte.

In the beginning of the era of ART, it was believed that the traditional sperm parameters could predict the capability to fertilise. However, as many couple experienced fertilisation failure in IVF (Lipitz *et al.,* 1993; Aboulghar *et al.,* 1996) or failed to obtain pregnancy, it became clear that the present markers of male fertility are not satisfactory. This is also reflected in ART results that has been more or less stable for the last couple of decades (Andersen *et al.,* 2005). The only parameter that has shown to be predictive of fertility is female age (Hull *et al.,* 1996). Also the fact that so many of the infertile couples remain undiagnosed (Evers 2002) and therefore are treated more or less blindly using one of the ART methods, has contributed to the urgent call for better fertility markers (Lewis 2007).

Until the 1990s, the majority of cases of severe male factor subfertility were virtually untreatable, and fertilisation failure was seen in a considerable number of IVF treatments. It was reported that up to 23% of the treatment cycles ended with no fertilisation after IVF, without any explanation for this (Lipitz *et al.,* 1993; Aboulghar *et al.,* 1996). Thus, the introduction of ICSI revolutionized the treatment of male factor infertility (Palermo *et al.,* 1992). However, since its introduction, ICSI has been a subject of an ongoing debate regarding its indications and safety (Govaerts *et al.,* 1996; Griffin *et al.,* 2003; Kurinczuk 2003; Verpoest and Tournaye 2006; Varghese *et al.,* 2007). Among the positive factors of ICSI are the

apparently low fertilisation failure rates compared with traditional IVF and the fact that the method has given men who previously were not able to be biological fathers a chance to conceive. More negative concerns have been raised related to technical, biological and genetic problems with ICSI. A number of reports have linked ICSI to an increased incidence of chromosomal anomalies, congenital abnormalities, imprinting diseases and perinatal hazards in offspring conceived with this technique (Fraga *et al.,* 1996; Ji *et al.,* 1997; Aitken and Krausz 2001; Cox *et al.,* 2002; Hansen *et al.,* 2002; Schieve *et al.,* 2002; DeBaun *et al.,* 2003; Orstavik *et al.,* 2003; Hansen *et al.,* 2005; Schieve *et al.,* 2005). One concern raised from studies on smokers whose ejaculates are under oxidative stress (OS) and characterized by high DNA fragmentation (Aitken and Krausz 2001), was an increased risk of childhood cancer in the offspring of smoking fathers (Fraga *et al.,* 1996; Ji *et al.,* 1997).

The aetiology of the increased risk of chromosomal anomalies in ICSI offspring, especially sex-chromosome anomalies, is thought to be partly multifactorial, partly andrological, related to paternal karyotypic abnormalities and/or abnormal sperm (Verpoest and Tournaye 2006). So far, follow-up studies in children born after ICSI compared with children born after conventional IVF have not been conclusive regarding the risks of congenital malformations and health problems in general (Kurinczuk and Bower 1997; Wennerholm *et al.,* 2000a; Wennerholm *et al.,* 2000b; Hansen *et al.,* 2002; Bonduelle *et al.,* 2003; Bonduelle *et al.,* 2004).

In addition to the criticism raised regarding hazards with ICSI, ART faces a problem of relatively low efficiency, as baby-take-home rates of 20-30% are usually reported (Andersen *et al.,* 2005). One of the reasons for this is a lack of adequate methods to evaluate the fertility potential of a couple and also lack of methods to find the most effective type of ART treatment for each couple. As no clear consensus or policies regarding indications for ICSI exist, the use of ICSI has increased substantially. Many laboratories now perform ICSI as their primary, if not only ART technique (Jain and Gupta 2007; Andersen *et al.,* 2008). Also couples without sperm defects request ICSI, and many are given clinical advice to proceed with ICSI, in situations where a few years ago traditional IVF would have been chosen and where it is reasonable to suppose that fertilisation rates would be as good as by ICSI (Bhattacharya *et al.,* 2001; Hamilton and Bhattacharya 2001).

For couples seeking ART, a more precise diagnosis could be helpful in order to identify the most optimal and less invasive ART treatment in a given case. ART is associated with high costs and a significant physiological burden (Schmidt 2006). So far, it has been the choice of each clinic to set criteria for IUI, IVF and ICSI. In order to meet patients' needs and to optimize fertilisation rates a dramatic increase in the use of ICSI has been seen, with all the possible negative effects discussed above (Jain and Gupta 2007; Andersen *et al.,* 2008).

A more precise diagnosing of subfertile men would also enable physicians to diagnose and counsel the infertile couple more optimal and could may also result in an extended use of cause-related therapy, which is very little used in today's clinical practice (Skakkebaek *et al.,* 1994). Examples of causes of subfertility in the male that can be treated pharmaceutically or by surgery include gonadotrophin treatment of hypogonadotropic hypogonadism (Howles *et al.,* 2007) and in cases of retrograde ejaculation, treatment with alpha-stimulating therapy (Kamischke and Nieschlag 2002) Vaso-vasostomy (Hendry 1994) and vaso-epididymostomy (Schoysman 1990) are applied in case of obstruction at vas deferens or epididymis level, respectively. Also different antioxidant treatments to reduce sperm DNA damage induced by reactive oxygen species (ROS) have been suggested (Greco *et al.,* 2005; Silver *et al.,* 2005; Menezo *et al.,* 2007). However, larger studies are needed to further refine the use and to confirm the results. Varicocele repair may also reduce sperm DNA damage, particularly, in those men with high levels of baseline sperm DNA damage (Zini *et al.,* 2005a; Werthman *et al.,* 2007). However, no clear consensus whether varicocele repair improves male factor fertility, and subsequently pregnancy rates exist (Marmar and Kim 1994; Lemack *et al.,* 1998; Nieschlag *et al.,* 1998; Marmar *et al.,* 2007).

Despite promising data provided by previous studies, the data concerning the role of sperm DNA integrity in ART needed to be further elucidated. The list of important questions remaining to be answered included: 1) Could ART bypass the biological mechanisms preventing sperms with DNA damage from fertilising an egg? 2) Would it be possible to define clinically applicable threshold values for sperm chromatin damage in raw and prepared semen in relation to the pregnancy outcome of ART? 3) Were fertilisation rates and embryo development in IVF and ICSI correlated to the level of sperm DNA fragmentation? 4) Was there any association between sperm DNA damage and pregnancy loss? 5) What was the level of intra-individual variation in regard to sperm chromatin integrity? Moreover, the underlying causes of fertilisation failure in IVF were unclear.

AIMS OF THE THESIS

The overall objective of this thesis was to identify genetic predictors of the outcome of assisted reproduction. Thereby, a more individualised and efficient infertility treatment could be offered to the couple.

The more specific aims were to evaluate:

- whether threshold values for DFI and HDS as measured by SCSA in relation to the pregnancy outcome of IUI, IVF and ICSI, could be obtained;
- whether there was a correlation between DFI or HDS values as measured by SCSA and fertilisation rates and cleavage stage embryo development in IVF and ICSI;
- whether there was an association between the SCSA parameters DFI and HDS and risk of pregnancy loss;
- how effective density gradient centrifugation could sort out DNA damaged spermatozoa and whether SCSA analysis of semen samples prepared by density gradient centrifugation could add more information in regard to the outcome of ART;
- whether DFI as measured by SCSA in an ART population is a more stable parameter in regard to intra-individual variation compared to the traditional sperm parameters;
- whether total fertilisation failure after *in vitro* fertilisation treatment could be explained by polymorphisms in the PCI gene or by increased sperm DNA fragmentation.

MATERIALS AND METHODS

Subjects

This thesis is based on a cohort of consecutive infertile couples who underwent ART at Viborg Hospital, Skive, Denmark during the period April 2002-December 2005. All men were given written as well as oral information and were asked to participate in a study of male reproductive function. All who accepted to be included in the study signed an informed consent. In total 998 ART cycles, from 637 couples were included in the cohort. All couples were treated with one of the three types of ART; IUI, IVF or ICSI. While most men were included in only one treatment cycle, other participated with up to 5 cycles.

All included men had a sperm concentration of at least 1 X 10^6 mill/ml. For the female partners, the inclusion criteria were: age <40 years; body mass index <30 and baseline follicle stimulating hormone (FSH) <12 IU/l.

In order to study whether there was true DFI or HDS threshold values for the pregnancy outcome of IUI, IVF and ICSI and if there was a correlation between DFI or HDS values and fertilisation rates and embryo development in IVF and ICSI, a pilot study based on 306 consecutive couples undergoing ART was conducted in the period of April 2002-March 2003 (Paper I). For each couple, only one treatment cycle during the study period was included in the analysis. Paper I was followed by further recruitment of patients and an extended study was performed (Paper II). During the period of April 2002– December 2003, in total 998 IUI, IVF and ICSI treatment cycles from 637 couples were included. Although sperm used for ART always undergo a preparation procedure, Paper I and II as well as most of the other SCSA-ART studies available have been based on studies of raw (unprepared) semen. In a total of 510 of the 998 samples density gradient centrifuged sperm were left after the ART procedure and could be analyzed with SCSA, on which the following study was based on (Paper III). Two hundred and eighty-two of the 998 patients were included in the study more than once, which gave basis for Paper IV, in which we studied the intra-individual variation of the SCSA parameters. Between 2 and 5 SCSA measurements were performed for each man corresponding to the ART treatments given to the couple.

In a subgroup of the entire study population (998 cycles); a group of 46 men involved in IVF cycles with fertilisation failure and 51 other men from IVF cycles with normal fertilisation rates (>50%), used as controls were included in a study on genetic polymorphisms as a cause of fertilisation failure (Paper V).

In figure 5, the study material is presented in a schematic form.

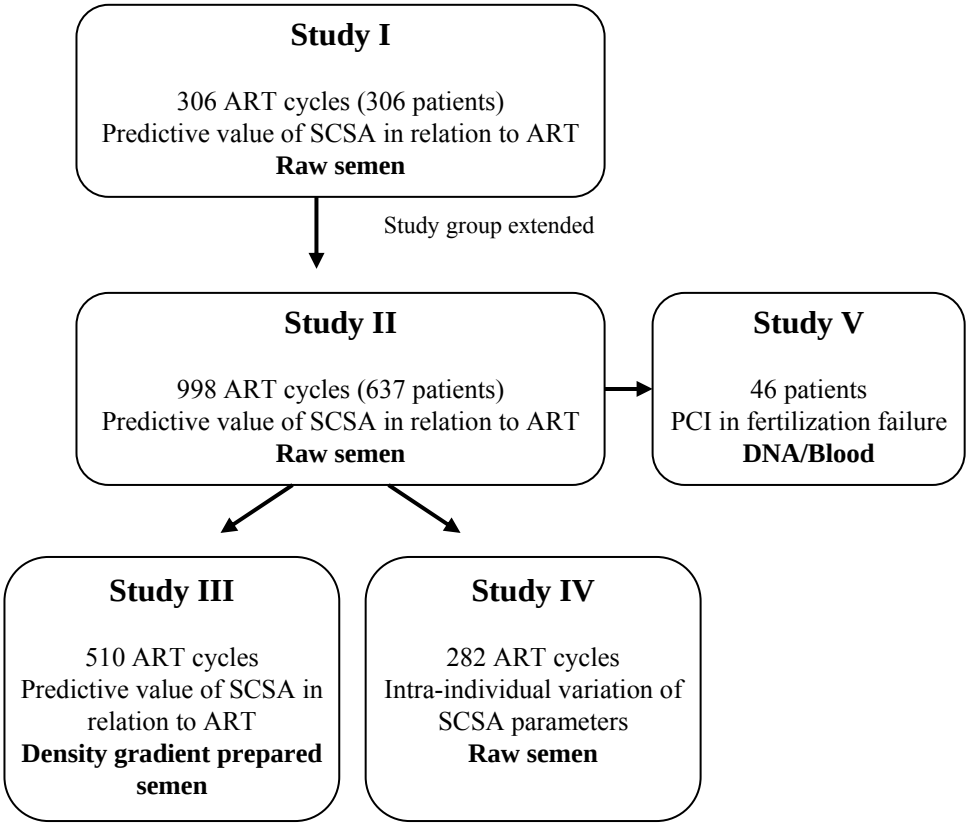

Figure 5. Schematic presentation of the five studies included in the thesis.

Methods

Collection and handling of semen and blood samples

Semen samples were collected by masturbation on the day of ovum-pick up or IUI. A sexual abstinence time of 2-5 days was recommended. One hundred microliters (µl) of the ejaculate was frozen at -80 °C, for subsequent analysis. At the same time, a blood sample for DNA analysis was collected in an EDTA-coated tube and frozen at -20 °C, for subsequent analysis.

As all genetic analyses were performed in Malmo University Hospital in Sweden, the frozen semen- and blood samples were transported by car from Skive, Denmark to Malmo on dry-ice. During the transport the samples were out of freezers for approximately 5 h.

Conventional sperm analysis

All semen samples were examined in the laboratory within thirty minutes after collection. Five µl of well liquefied semen was placed on a Makler–chamber. All measurements were as performed on a Nikon phase contrast microscope on a heating stage (37˚C) at a total magnification of x 40. Sperm concentration was assessed by using undiluted semen. The number of spermatozoa counted in any strip of 10 squares of the grid of the Makler-chamber indicated their concentration in million/ml. A mean of 10 x 2 squares was calculated. Motility was scored according to the WHO guidelines (WHO, 1999). Sperms were categorized in types A, B, C and D. Type A corresponded to rapid progressive motility, B to slow progressive motility, C to non-progressive motility and D represented immotile sperm. Sperm morphology was not assessed.

Analysis of sperm DNA fragmentation

Currently, there are three major tests of sperm DNA fragmentation, including the Comet assay (single cell gel electrophoresis) (Morris *et al.,* 2002), the TUNEL (terminal deoxynucleotidyl transferase-mediated dUDP nick end labelling) assay (Gorczyca *et al.,* 1993) and the sperm chromatin structure assay (SCSA) (Evenson *et al.,* 1980, Evenson *et al.,* 2002). Comet, TUNEL and SCSA all label single or double stranded DNA breaks. Whilst Comet is a fluorescence microscopic test, TUNEL can be applied in both bright field/fluorescence microscopy and by flow cytometry. In Comet assay sperm cells are mixed with melted agarose and then placed on a glass slide. The cells are lysed and then subjected to horizontal electrophoresis. DNA is visualized with the help of a DNA specific fluorescent dye and DNA damage is quantified by measuring the displacement between the genetic material of the nucleus comet head and the resulting tail. In the TUNEL assay, terminal deoxynucleotidyl transferase (TdT) incorporates labelled (by and large. Fluorescent) nucleotides to 3'-OH at single and double-strand DNA breaks to

create a signal, which increases with the number of DNA breaks. The fluorescence intensity of each scored sperm is determined as a ‘‘positive’’ or ‘‘negative’’ for sperm on a microscope slide. In a flow cytometer the fraction of positive sperm is represented by the cells above a threshold channel value on a relative fluorescent intensity scale.

In the current studies SCSA was chosen for the studies of sperm DNA fragmentation. The SCSA is a flow cytometric based method, with the clear benefit of analyzing 5-10 000 cells compared to 2-300 cells usually analyzed in bright field and fluorescence microscopy. In the early 1980s, Evenson and co-workers (Evenson *et al.,* 1980) described the sperm chromatin structure assay (SCSA) and the method was later refined (Evenson *et al.,* 2002). The SCSA utilizes the metachromatic properties of the fluorescent stain acridine orange (AO), and the extent of DNA denaturation after an acidic treatment is determined by measuring the shift from green fluorescence (double-stranded, native DNA) to red fluorescence (single-stranded, denatured DNA). Following the flow cytometric analysis data are further analyzed using dedicated software (List View, Phoenix Flow Systems, San Diego, CA or SCSASoft; SCSA Diagnostics, Brookings, SD, USA). Computer gates are used to determine the proportion of spermatozoa with increased levels of red fluorescence (denatured single-stranded DNA) and green fluorescence (native double-stranded DNA). The extent of DNA denaturation is expressed in terms of the DNA fragmentation index (DFI), which is the ratio of red to total (red plus green) fluorescence intensity, i.e. the level of denatured DNA over the total DNA (Evenson *et al.,* 2002). The DFI value is calculated for each sperm cell in a sample, and the resulting DFI frequency profile is obtained (Figure 6, right, upper panel). Most sperm form a unimodal distribution representing the normal population of sperm with no detectable DNA damage. Sperm with higher red fluorescence, falling in the histogram area beyond the curve of normal sperm, represent the population of abnormal sperm with detectable DFI. The fraction of high DNA stainable (HDS) cells (immature spermatozoa) is calculated by setting an appropriate gate on the bivariate cytogram (Figure 6, right, lower panel) and considering as immature spermatozoa those events which exhibit a green fluorescence intensity higher than the upper border of the main cluster of the sperm population with a non detectable DFI. HDS are thought to represent immature spermatozoa with an incomplete protamination (Evenson *et al.,* 2002).

For the flow cytometer set-up and calibration, a reference sample was used from a normal donor ejaculate sample retrieved from the laboratory repository. The intra-laboratory coefficient of variation was found to be 4.5% for DFI and 10% for HDS, respectively. For the flow cytometer set-up and calibration, reference samples were used from a normal donor ejaculate sample retrieved from the laboratory

repository. A total of 5000 (Papers I, II, IV and V) –10 000 events (Paper III) were accumulated for each measurement at a flow rate 200–300 cells/s.

SCSA measurements were performed on raw semen (Papers I, II, IV and V) and on density gradient centrifuged semen (Paper III).

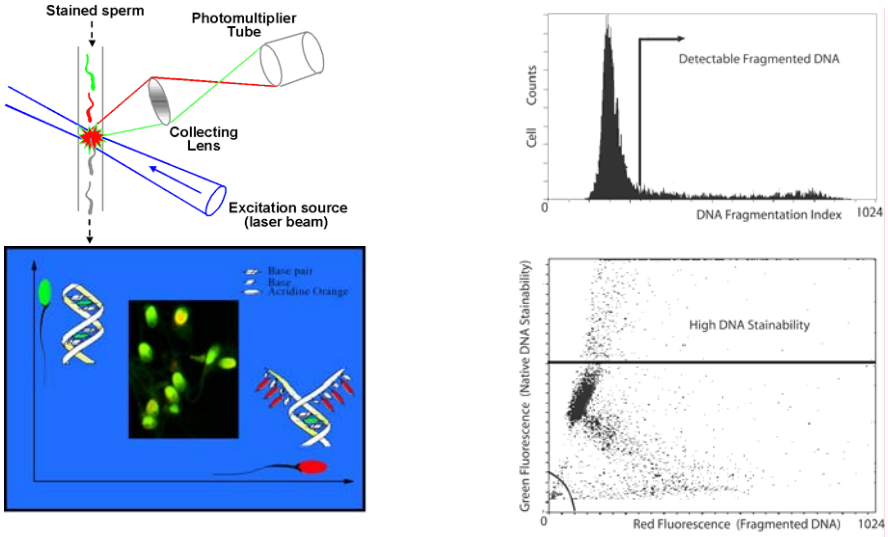

Figure 6. Left: Principles of flow cytometry and SCSA**.** Right: Histo- and cytogram for DNA fragmentation index (DFI) and High DNA stainability (HDS).

The SCSA is a standardized test (Evenson *et al.*, 2002). Apart from being subject to a very limited intra-laboratory variation (Giwercman *et al.*, 1999), however, the SCSA analysis has shown to be very robust to variation between laboratories. In an external quality control based on >180 samples, a high (r = 0.8) correlation was found between the values obtained by our laboratory and those from a control laboratory. Furthermore, not only was there a high level of correlation between the results reported by two independent laboratories that strictly followed the SCSA protocol, but the absolute DFI values obtained at two different places, using different equipment, did not on average differ by >1% (Giwercman *et al.*, 2003).

Testing with SCSA has several advantages: it evaluates a high number of sperm in a short period of time; 5-10 000 cells compared to most light microscopic tests where 1-300 cells normally are analyzed. When first have done the flow-cytometer

set up for the day, it is a fast method of screening multiple samples. This test uses frozen or fresh samples, so the analysis can be planned in due time.

A disadvantage with SCSA is that relatively expensive equipment; a flow cytometer is required. Also the fact that sperm samples with very low concentration cannot be analysed is negative. Very few flow cytometric protocols are as demanding as the SCSA and Boe-Hansen *et al.* (Boe-Hansen *et al.,* 2005) demonstrated that small deviations from the protocol could impact the result of the test. Good quality routines are of great importance. Moreover, the test irreversibly damages sperm, they cannot be used for fertilisation.

Analysis of mutations and polymorphisms

A single nucleotide polymorphism (SNP) is the most common form of polymorphism in the human genome, representing about 90% of all known genetic variation. SNPs arise due to errors in the DNA replication and repair, and consist of a change in a single nucleotide (A, T, C or G). For a variant to be considered as a SNP, it must occur in at least 1% of the population. SNPs can occur in coding as well as non-coding or in intergenic regions of genes and can lead to changes in the protein that is produced. SNPs located in coding sequences do not necessarily lead to change of an amino acid sequence of the protein, but may still have consequences for gene splicing, transcription factor binding or the sequence of non-coding ribonucleic acid (RNA). Only 3-5% of the DNA codes for proteins. Studies have estimated that a common SNP occurs approximately every 600 basepair (bp) (Kruglyak and Nickerson 2001), mostly found in non-coding regions.

A number of methods to screen for polymorphisms or point mutations in a specific gene exist. Amplification by polymerase chain reaction (PCR) (Mullis and Faloona 1987) detect major structural defects, such as large deletions, by failure of amplification of the correct fragment. Techniques as denaturing gradient gel electrophoresis (DGGE) (Myers *et al.,* 1985) or single-strand conformation polymorphism (SSCP) analysis (Orita *et al.,* 1989) have been developed to screen for small insertions or single base deletions within a gene. In order to precisely characterize mutants, however, sequence analysis is required (Wong *et al.,* 1987; Ward and Coffey 1991).

In the present study, amplification was performed by PCR followed by direct sequencing (Paper V). Having isolated a target gene, PCR is used to extend the number of copies of a gene which is necessary to have enough starting template for sequencing and further analyses of gene-specific mutations. PCR is based on three distinct steps: DNA denaturation, primer annealing and primer extension, all governed by changes in temperature (McPherson 2000). By repeating these steps in a cyclic manner, the result is more than one million copies of the target gene.

ART-procedures

While the least invasive form of ART, IUI, in which prepared semen is inseminated to the women's uterus, is used in ovulatory dysfunction, unexplained subfertility and milder forms of male subfertility, IVF is primarily used in female subfertility or as a second choice in unexplained causes (Edwards and Brody 1995). Originally, ICSI was developed to be used in severe male infertility cases, however is now increasingly used for all types of infertility/subfertility (Jain and Gupta 2007; Andersen *et al.,* 2008). In IVF, oocytes are fertilised by sperm *in vitro*. The process involves hormonal stimulation and ovum pick-up. Two to five days later the fertilised and cleaved embryo is transferred to the patient's uterus. For ICSI the same treatment principles are followed, however, here one single spermatozoon is injected directly into the cytoplasm of the oocyte.

In a vast majority of cases, spermatozoa used for ART are, prepared by density gradient centrifugation (DGC) in order to favour the isolation of motile and morphologically normal spermatozoa. The media used for DGC consists of a colloidal silica suspension in a culture medium. After layering of semen on the top of the two layer gradient (45 and 90%), the sample is centrifuged at 300 g for 15 min. Thereafter the pellet is washed and diluted in culture media used for fertilisation. The system separates normal sperm from lymphocytes, epithelial cells, abnormal or immature sperm, cell debris, bacteria and seminal fluid.

All ART procedures followed standard ART regimes as described in Papers I-III.

In our studies, IVF/ICSI patients received embryo transfer of one or two embryos on day 2 (60% of the cycles), on day 3 (38.5% of the cycles) or on day 5, at the blastocyst stage (1.5% of the cycles). A biochemical pregnancy (BP) was defined by a plasma β-hCG concentration >10 IU/l. A clinical pregnancy (CP) was defined as an intrauterine gestational sac with a heart beat 3 weeks after a positive β-hCG-test. The implantation rate was calculated as the ratio of gestational sacs determined by ultrasound after 7 weeks in relation to the total number of embryos transferred.

Statistical analysis

Statistical analysis was performed by the use of SPSS for Windows, versions 11.0, 11.5 and 14.0 (SPSS Inc., Chicago, USA) (Studies I-IV). For Fisher's exact test, the site www.graphpad.com was used (Studies I, III and V).

Two-tailed tests were performed in all studies. A two-tailed test is appropriate when the researcher has no a priori expectation regarding the values he observes. With any significant deviation from the reference value, the null hypothesis is rejected. The term 'statistically significant' was used to denote a two-sided p-value <5%.

Coefficient of variation (CV) was calculated using the formula (SD/mean) × 100% (Study IV).

Risk assessments in Studies I and II were performed by the use of binary logistic regression analysis, expressed as odds ratio (OR) with 95% confidence interval (CI).

Linear regression analysis was in Study III used to compare SCSA parameters for those who achieved pregnancy and those who did not. In Study IV, linear regression analysis was used to compare CV between groups.

Fisher's exact test was used in Studies I, II, III and V. Fisher's exact test is used to examine the significance of the association between two variables in a 2x2 contingency table, and can even be applied when the cell sample size is <5 (Study V).

Mann Whitney test was applied to test inter-group differences (Study I). The Mann-Whitney test is a non-parametric test that is used for comparison of means and distributions of two independent groups when data do not meet the normal distribution requirements for a t-test. It is an alternative to the t-test, almost as powerful as the t-test in detecting a true difference when the residuals follow a normal distribution.

In Study II, male and female age, male and female BMI, smoking habits, sperm concentration, motility and treatment number were considered as potential confounders, all tried in the model according to the change-in-estimate method suggested by Greenland (Greenland 1989), using a 10% change for inclusion and a 5% change for exclusion. The same factors, dichotomized at their respective medians, were also tested as effect modifiers, using the Breslow–Day test for homogeneity.

Spearman´s rho was in Study I and IV used to calculate binary correlations between parameters.

RESULTS AND DISCUSSION

Sperm DNA fragmentation and outcome of ART

The main focus of the studies leading to Papers I-III has been to identify whether the sperm chromatin structure assay (SCSA) parameters DFI and HDS could predict the outcome of assisted reproduction. Data regarding pregnancy, delivery and miscarriage for IUI, IVF and ICSI as well as fertilisation and embryo development for IVF and ICSI will be presented and discussed in the following.

Predictive value of SCSA in intrauterine insemination

The most important and novel finding of the present studies is that DFI can be used as an independent predictor of fertility in couples undergoing IUI (Papers I and II).

The first indications of the high predictive value of SCSA regarding the outcome of IUI treatments were found by combining the two SCSA parameters, DFI and HDS (Paper I). The chance of pregnancy and delivery for IUI was significantly higher in the group having a DFI below 27% and a HDS below 10% compared to patients with a DFI>27% or a HDS>10%. In the group with a DFI≤27% or a HDS≤10%, the odds ratios (ORs) (95% Confidence Interval (CI)) were 20 (2.3-117), 16 (1.9-137) and 14 (1.6-110) for biochemical pregnancy (BP), clinical pregnancy (CP) and delivery (D), respectively. In figure 7, the DFI 27% threshold is called "Evensons threshold (ET)" since this threshold level was suggested by his research group (Larson *et al.,* 2000; Larson-Cook *et al.,* 2003).

After extending the study population (Study II), and in opposite to study I, by using those patients with DFI<30% as a reference group, the ORs for BP, CP and D were significantly lower for couples with DFI>30%, referred to as the Bungum´s threshold (BT) (Figure 7). The ORs (95%CI) were 0.10 (0.02±0.42), 0.10 (0.02±0.042) and 0.07 (0.01-0.048) for BP, CP and D, respectively. In contrast to study I, HDS was not of predictive value for the outcome of IUI, neither alone nor in combination with DFI (Paper II).

With 387 IUI cycles, our study is so far, the largest SCSA study. Whilst the proportion of children born per cycle was 19.0 % when the DFI value was below 30%, those with a DFI value above 30% only had a take-home-baby rate of 1.5 %. Although a very clear cut-off level was seen for DFI (Figure 7), it is important to point out that a DFI above 30% does not preclude a normal pregnancy; however, the likelihood of obtaining a pregnancy by IUI is, almost non-existing. The highest DFI levels that gave rise to pregnancies by IUI were 34% and 42.1%, the former resulted in a live birth, whereas the latter resulted in a spontaneous abortion (Paper II).

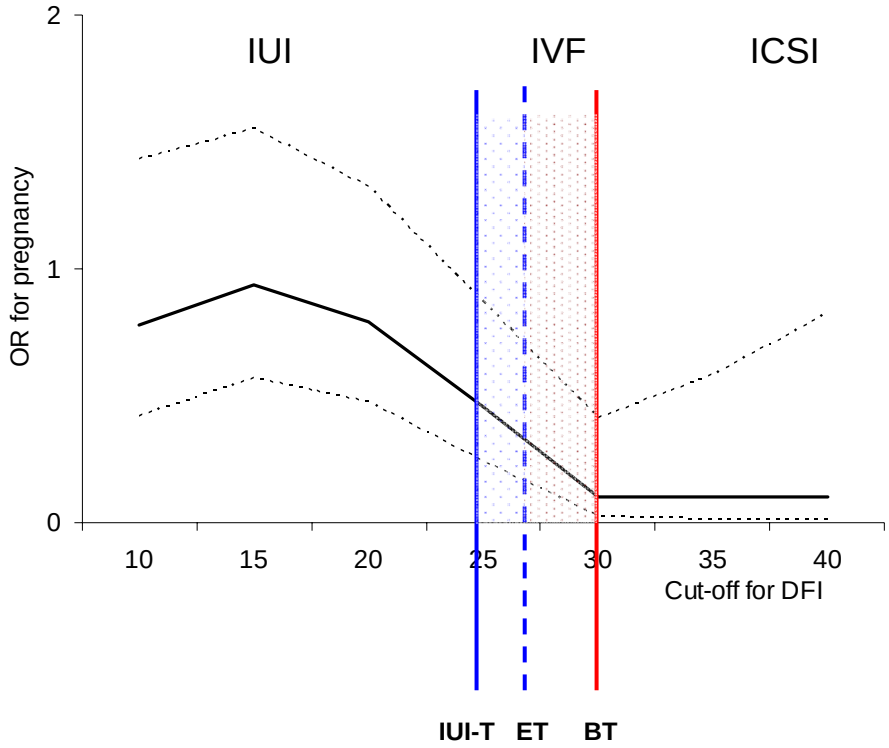

Figure 7. Odds ratios (OR) for biochemical pregnancy in relation to DNA fragmentation index (DFI) following Intrauterine insemination (IUI), visualising the three cut-off thresholds suggested; IUI-threshold (IUI-T), Evenson´s threshold (ET) and Bungum´s threshold (BT).

The first study to indicate an association between DNA damage and reduced pregnancy chances was the study by Duran *et al.* (Duran *et al.,* 2002). In a retrospective study including 119 couples (154 IUI cycles) the authors concluded that pregnancy could not be achieved when DNA fragmentation, as measured by the TUNEL assay, was above 12 %. Similar findings have been reported by others. Saleh *et al.* (2003a) performed a small study where 12 of 19 couples had a DFI value as measured by SCSA above 28 % and all these couples were unable to achieve a pregnancy. More recently, Boe-Hansen *et al.* (2006) used the SCSA in a study on 48 couples undergoing IUI treatment. Only two of these couples had a DFI value above 30%, and did not achieve any pregnancy.

In summary, results from Studies I and II have clearly shown that DFI can be used as an independent predictor of fertility in couples undergoing IUI.

The clinical recommendation drawn from these data is that SCSA should be implemented in the infertility work-up and used as a tool to decide which type of ART-treatment that should be performed. In our region, the SCSA is now one of the criteria for the reimbursed ART treatment, used as a supplement to the traditional sperm analysis. Although a clear cut-off value corresponding to DFI = 30%, could be set, we found that the chance of obtaining a pregnancy by IUI decreased successively from about DFI=20%. Taking into consideration the rapid decline in the probability of pregnancy by IUI with an increase in DFI above 20%, we have chosen to set a 25% IUI-threshold (IUI-T) in our clinic. In other words, if a man presents with a DFI below IUI-T and all other criteria for IUI are fulfilled (≥ 5 mill. progressive motile sperm following gradient centrifugation, female age <36 years and normal fallopian tubes), IUI is chosen as the first line treatment. In cases of DFI between 25 and 30%, the couple is referred to IVF treatment and finally, if DFI is above 30%, ICSI is recommended (Figure 7).

Predictive value of SCSA in IVF and ICSI

Another aim of the studies was to elucidate whether SCSA could be used in prediction of IVF and ICSI results. Some of the first SCSA-ART studies (Larson *et al.,* 2000; Larson-Cook *et al.,* 2003) suggested that no pregnancy could be obtained for DFI above 27%, regardless of the type of ART applied. In our initial study (Paper I) we therefore used this level in our calculations. In contrast to Larson's findings (Larson *et al.,* 2000; Larson-Cook *et al.,* 2003), we demonstrated that a DFI level above 27% is compatible with pregnancy and delivery after both IVF and ICSI (Paper I and II). Hence, ART is able to compensate for the impairment of sperm chromatin integrity, in particular if ICSI is chosen as fertilisation method.

In Study I, the likelihood of obtaining a CP was found to be the same in the group of patients with DFI above 27%, as in those with DFI below 27% (CP= 38% in both groups) (Paper I). The implantation rates were also comparable, 31% versus 34%, respectively.

Comparing the two in vitro fertilisation methods; IVF and ICSI, no statistical difference between the pregnancy outcomes was observed in the group with DFI below 27%. However, in the group with DFI above 27%, the results of ICSI were significantly better than those of IVF with respect to BP (71% versus 22%; p = 0.007) as well as CP (53% versus 22%; p = 0.09). For D rates, the proportions were 47% in the ICSI and 22% in the standard IVF group (p = 0.16) with an OR=7.8 (95% CI 1.7±36) for BP, OR=3.9 (95% CI 0.9±17.0) for CP and OR=3.1 (95% CI 0.72±13) for D. After inclusion of sperm concentration, proportion of progressively motile sperm, female age and treatment number in the model, the ORs for positive reproductive outcome when ICSI was compared with IVF increased to

26 (95% CI 1.9±350), 7.7 (95% CI 0.84±70) and 7.0 (95% CI 0.75±65), with BP, CP and D dependent variables, respectively (Paper I).

In Study II, further data analyses included the use of different thresholds (5%, 10%, 15%, etc.) to establish the possible presence of a threshold effect (Paper II). These results indicated that 30% was the most suitable threshold point to use for the main analyses (study II). No statistically significant difference was seen between the outcomes of ICSI versus IVF in the group with DFI≤30%. In the DFI>30% group, however, the results of ICSI were significantly better than those of IVF. The ORs for BP, CP and D were 3.0 (95% CI: 1.4–6.2), 2.3 (5% CI: 1.1–4.6) and 2.2 (95% CI: 1.0–4.5), respectively. For ICSI, there was even a tendency towards higher rates of BP, CP and D with a DFI>30% versus a DFI≤30%, however this was not a statistical difference. Moreover, the implantation rate in the ICSI group with DFI>30% seemed to be higher than in any other subgroup (Paper II).

As already shown for IUI, HDS did not predict the outcome of IVF or ICSI, neither alone nor in combination with DFI (Papers I and II).

Our results have been confirmed by several others (Gandini *et al.,* 2004; Virro *et al.,* 2004; Check *et al.,* 2005; Boe-Hansen *et al.,* 2006). However, a common problem for all more recent studies was the rather limited number (36-42 IVF/ICSI) of patients. Moreover, no potential confounders (e.g. female age) were introduced in the analysis of data. In our study, although with a marginally effect on the outcome parameters, male and female age, male and female BMI, smoking habits, sperm concentration and motility and treatment number were considered as confounders (Paper II). One single study consisting on 100 IVF/ICSI treatments, has reported that DFI and HDS threshold values were not valid (Payne *et al.,* 2005). More recently, some meta-analyses on sperm DNA damage as measured by the SCSA and ART outcomes were published (Evenson and Wixon 2006; Li *et al.*, 2006; Collins *et al.,* 2008). The first study showed that in IVF and ICSI, clinical pregnancy was closely related to DFI as measured by SCSA. In contrast, Li *et al.* found that neither DFI nor HDS had an effect on the chance of CP after IVF or ICSI treatment. Both reports included IVF and ICSI data from our Paper I. In the third and most recent metaanalysis (Collins *et al.,* 2008), where also our data from study II were included, a small but statistically significant association between sperm DNA integrity test results and pregnancy in IVF and ICSI cycles were found. The different conclusions drawn by the two studies are most likely due to use of different inclusion criteria.

Previously the efficacy of IVF and ICSI was found to be equal in cases of non-male factor infertility (Bhattacharya *et al.,* 2001). As shown for IUI (Paper I and II), normal sperm chromatin integrity becomes particularly important when the contact between the two gametes occurs in a natural way. Others have suggested

that, selective pressures operate to avoid the development of an embryo derived from sperm with a high load of genetic in a natural environment (Morris *et al.,* 2002). This could be different in ICSI, where a sperm is forced into the oocyte and the possibility of selecting a genetically defective sperm is much higher. ICSI enables fertilisation to take place in spite of severely compromised semen characteristics and DNA damage (Ahmadi and Ng 1999a). This is illustrated by the fact that even ICSI performed with testicular round spermatids, whose chromatin structure by definition is quite different from that acquired by sperm after spermiogenesis and epididymal maturation, can give rise to successful pregnancies (Mansour *et al.,* 2003).

No directly documented biological explanation for why ICSI is superior to IVF when DFI exceeds 30% is presented, neither by us (Papers I and II) nor by others. In Paper II we suggested two possible explanations for this. Our first point was that women in the ICSI group, on average were slightly younger than women in the IVF group. In the ICSI group, infertility was mainly caused by male factor, and thus the younger women may produce oocytes with a better DNA repair capacity. Secondly, we suggested that the difference in favour of ICSI might also be due to the two completely different culture environments used for IVF and ICSI. While IVF oocytes were exposed to spermatozoa for 90 min, in ICSI the spermatozoon was injected directly into the oocyte and therefore probably less exposed to ROS than in IVF.

Recently, a number of works that may indirectly support our suggestions regarding ROS created in IVF culture but not in ICSI as a possible explanation for the difference in success rates between ICSI and IVF have been published. A group of Australian researchers demonstrated that high levels of estrogenic compounds caused oxidative stress, which led to DNA damage in human spermatozoa (Bennetts *et al.,* 2008). In the IVF environment not only the oocyte and the sperm are present, but also the cumulus complex consisting of a high number of corona- and granulosa cells is a natural part of the culture. In contrast, in the ICSI environment, all corona- and granulosa cells are chemically and mechanically removed. It may be speculated that sperms with high DFI are more vulnerable to the adverse effects of ROS or that cumulus and corona cells surrounding the egg release estradiol (Kattera and Chen 2003), which in turn has been shown to have a direct toxic effect on the embryo (Valbuena *et al.,* 2001).

The difference seen within the ICSI group, in favour of the group with DFI >30% is even more difficult to explain. A possible superiority of ICSI oocytes might be most pronounced at the highest DFI levels at which natural conception is not possible despite excellent fertility status of the female.

In summary, we have demonstrated that when DFI was above 30%, higher pregnancy and birth rates were found after ICSI compared to IVF. In contrast, no differences between the two methods were seen when DFI was below or equal to 30%. Similarly to the findings in the IUI group, HDS were not shown to be of predictive value for the outcome of the treatment, neither alone nor in combination with DFI (Papers I and II). However, it should be kept in mind that although study I and II might indicate that ICSI is superior to IVF in case of high DFI, final conclusions need to be based on studies where couples with high DFI and otherwise fulfilling the criteria for IVF are randomised to IVF and ICSI.

In the Fertility Clinic, Viborg Hospital, Denmark, where the patient material for the present studies was collected, the results of the SCSA-measurements used for the studies in this thesis were not applied clinically. Some of the patients had a history of one or more IUI cycles prior to study inclusion. If the patient not obtained pregnancy in the actual study cycle, some also proceeded with further IUI, IVF or ICSI treatment or they dropped out. Thus, we found it interesting to examine the patient records to see the outcome for those patients having a DFI above 30% and not becoming pregnant in the actual study-cycle. As some of the patients were included in their first treatment cycle and others in one of the subsequent cycles, we found it interesting to look both forward and backwards in time in order to get full information about the outcome. We therefore registered cycle outcome data for the total number of ART cycles these patients underwent.

In 51 patients, a total of 208 IUI cycles were performed (Bungum, unpublished data). Four singleton pregnancies resulting in 2 deliveries were obtained (<1%), why further treatment in these women was terminated (Table 6). Thirty eight patients dropped out and regarding these women no information about other fertility treatments or spontaneous pregnancies was further available. Eleven patients proceeded with either IVF or ICSI in the clinic (in total 21 treatment cycles). Among these 11 women, 10 pregnancies resulting in 9 deliveries were obtained during their in total 21 treatment cycles. By a separate analysis of the IVF and ICSI groups, there was a tendency of a better pregnancy rate in the ICSI group compared to the IVF group.

Table 6. Reproductive outcomes for patients with DFI above 30%. While some dropped out after 1-5 IUI-cycles, others proceeded with more advanced ART: IVF or ICSI.

	Number of patients	Total number of cycles	Mean female age	Mean DFI, %	Number of clinical pregnancies	Number of deliveries
IUI	51	208	32.3	42.6	4 (1.9%)	2 (0.85%)
Proceeded to IVF	4	8	32.3	44.0	3 (37.5%)	2 (25%)
Proceeded to ICSI	7	13	31.1	47.2	7 (53.8%)	7 (53.8%)

The number of patients included in the follow-up, which is a retrospective clinical report rather than a study, is limited. However, the outcome data are convincing and completely in line with our published data (Papers I and II).

SCSA parameters in relation to fertilisation and embryo development

Comparing fertilisation and embryo quality (cleavage developmental stage and fragmentation rate) between those having a DFI>30% and those having a DFI≤30%, no statistically significant differences were seen, neither for IVF nor for ICSI patients (Paper II).

Our findings are in accordance with most other previous and recent papers using the SCSA analysis (Larson *et al.,* 2000; Larson-Cook *et al.,* 2003; Gandini *et al.,* 2004; Virro *et al.,* 2004; Li *et al.,* 2006) as well as other sperm DNA integrity testing methods (Sakkas *et al.,* 1996; Hammadeh *et al.,* 2001; Tomlinson *et al.,* 2001; Morris *et al.,* 2002; Tomsu *et al.,* 2002; Henkel *et al.,* 2004; Lewis *et al.,* 2004; Huang *et al.,* 2005; Nasr-Esfahani *et al.,* 2005). In contrast a few investigators have shown a negative correlation between sperm DNA fragmentation and IVF and ICSI fertilisation rates (Sun *et al.,* 1997; Lopes *et al.,* 1998a; Saleh *et al.,* 2003a; Payne *et al.,* 2005). Host *et al*. (Host *et al.,* 2000a) determined the incidence of spermatozoa with DNA strand breaks with the Tunel assay in different groups of infertile couples, finding negative correlations between the proportion of spermatozoa with DNA strand breaks and the fertilisation rates in all groups except for these undergoing ICSI. In the study by Virro *et al.* (2004), HDS was found to be related to IVF but not to ICSI fertilisation rates. This made

the authors suggest that men with HDS>15% should be treated with ICSI. Their finding has, however, not been confirmed by others.

Several factors might contribute to the discrepancy between the studies regarding the association between sperm DNA parameters and fertilisation rates. There is no clear consensus on policies regarding indications for IUI, IVF and ICSI, and therefore the selection of patients may differ from study to study. Furthermore, different techniques for the assessment of sperm DNA integrity were applied and although there is a correlation between the results obtained by these assays, the correlations were only moderate (Gorczyca *et al.,* 1993; Aravindan *et al.,* 1997; Erenpreiss *et al.,* 2004), indicating that they are assessing different biological phenomenons. Even the definition of fertilisation may differ from laboratory to laboratory.

Ahmadi and Ng who in a mouse model demonstrated the DNA-damaged spermatozoas ability to fertilise the oocyte (Ahmadi and Ng 1999a), also reported sperm DNA damage to be related to poor embryo development (Ahmadi and Ng 1999b), although the oocyte, to a certain degree, was able to repair the sperm DNA damage. The human ART-data regarding embryo development in relation to DNA damage is, however, somewhat conflicting. In accordance with our findings, several other authors have reported similar cleavage stage embryo developmental rates between high and low DFI groups (Larson *et al.,* 2000; Larson-Cook *et al.,* 2003; Gandini *et al.,* 2004; Boe-Hansen *et al.,* 2005; Payne *et al.,* 2005). This, however, is contrasted by the findings of others (Sun *et al.,* 1997; Morris *et al.,* 2002; Saleh *et al.,* 2003a), who showed that DFI levels were negatively correlated with embryo quality after IVF and ICSI. Seli *et al.* (2004) and Virro *et al.* (2004) reported that men with high levels of DNA fragmentation were at increased risk of low blastocyst formation rate compared to those with a low DFI. Studying embryo development in a bovine model, Fatehi *et al.* (2006) suggested that damaged gamete DNA does not affect embryo development until after the first cleavage, when embryonic gene expression begins, due to the fact that before the first 2–3 cleavages, the mRNA still present from the cytoplasm of the gametes is being translated instead of waiting for the embryonic DNA to be transcribed (Braude *et al.,* 1988). However, when embryonic gene expression is first initiated during the second or third cleavage, one can observe the formation of an abnormal mitotic spindle. DNA fragmentation also occurs at that point, and Fatehi et al. claimed that it can generate severe developmental problems as the embryo attempts to form a blastocyst (Fatehi *et al.,* 2006). The results showed that sperm DNA damage did not impair the first 2–3 cleavages, but blocked blastocyst formation by inducing apoptosis.

On the basis of the impaired blastocyst development claimed to be caused by sperm DNA damage, it has been suggested to routinely culture to the blastocyst

stage (Spano *et al.,* 2005a). So far, due to the lack of adequate blastocyst culture media that could support in vitro embryo development, day 2 and 3 embryo transfers have been the dominant procedure. Prolonging the culture may allow normal embryos to develop to the blastocyst stage and permit the selection of embryos that have the potential of continued development under embryonic genome control rather than embryos that carry aberrant (paternal) DNA. Although this could seem logic, from a biological point of view, the arguments for such a practice could be discussed. In a previous work (Bungum *et al.,* 2003), in which day 5 and day 3 (cleavage stage) embryo transfer were compared in a randomized manner, no differences between day 3 and day 5 transfers were observed as regards embryo development, implantation and pregnancy rate. However, when dividing patients into groups according to fertilisation method: IVF or ICSI, in the group of patients in whom ICSI was performed, a significantly lower blastocyst formation rate and implantation rate compared to the IVF group was seen, 51% versus 60% and 28% versus 47%, respectively. On the other hand, no statistical difference was seen in pregnancy rates. Unfortunately, no SCSA data were available from these patients. However, from the studies presented in this thesis it is evident that significantly higher DFI levels are found in ICSI patients compared to the IVF group and it can be speculated whether this is related to the lower blastocyst formation rate in the former group of patients.

SCSA parameters and risk of miscarriage

The present study showed no statistically significant association between high DFI or HDS and early pregnancy loss (Paper II). Despite this, it cannot be excluded that DFI levels >60% are associated with a higher risk of early pregnancy loss (Figure 8). Even a material including about 1000 ART treatments, in which Paper II was based on, is small when it comes to reaching firm conclusions in this question. Only 6 of the patients who gave rise to a pregnancy by IUI, IVF and ICSI had a DFI above 60%.

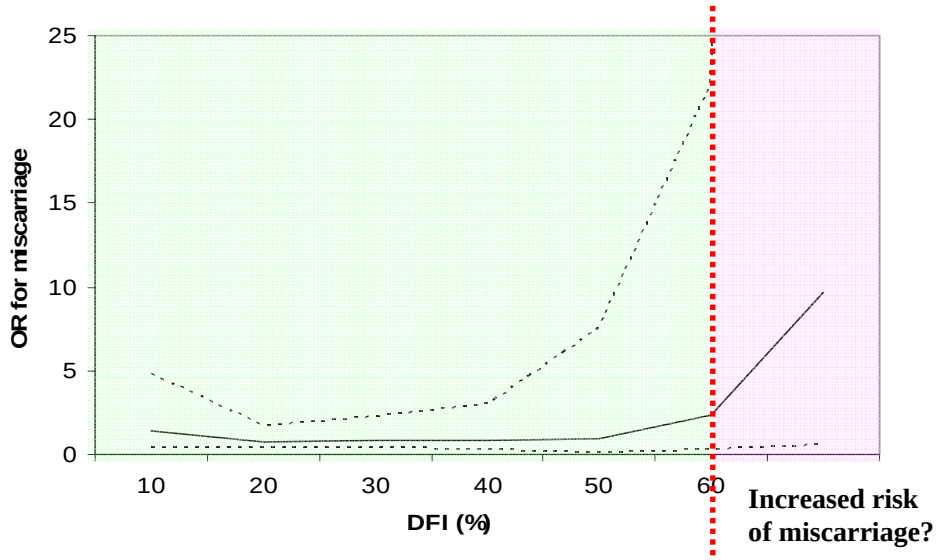

Figure 8. Odds ratios (ORs) for pregnancy loss in relation to threshold levels for the DNA fragmentation index (DFI), all three treatment categories, IUI, IVF and ICSI combined. The threshold for a possible increased miscarriage risk (DFI>60%) is shown.

A number of recent, smaller studies (including 60-322 patients) have reported an association between increased (albeit non-significant) risk of pregnancy loss after IVF and/or ICSI and a high DFI (Virro *et al.,* 2004; Check *et al.,* 2005; Zini *et al.,* 2005b; Borini *et al.,* 2006; Lin *et al.,* 2007; Frydman *et al.,* 2008). Lin *et al.* (2007) also found that in couples treated with IVF, in whom the male partner had a HDS above 15%, significantly higher IVF miscarriage rates were seen compared to couples where the HDS was below 15%. The authors, therefore, recommended ICSI in men with HDS>15%.

An association between sperm DNA damage and pregnancy loss has also been observed in non-ART studies. In the Georgetown study, Evenson *et al.* (1999) observed an increased risk of pregnancy loss in couples with sperm DNA damage. Also Carrell *et al.* (2003) reported that recurrent pregnancy loss is associated with higher levels of sperm DNA damage.

Based on the conflicting results seen in existing human studies, one could suspect that a subgroup exists within the high DFI-population (related to the type and aetiology of DNA damage) with an increased risk of miscarriage. New larger scale studies allowing further stratification into groups due to the type and aetiology of DNA damage should be performed.

Raw versus prepared semen

In Studies I, II, IV and V, all SCSA analyses were performed on raw semen as in most other SCSA-ART studies published to date. In Study III, SCSA data from both raw and density gradient centrifuged (DGC) semen of 510 of the 998 ART cycles representing the study II population were analyzed.

Contrasting what was concluded for raw semen, no predictive value of the SCSA parameters DFI and HDS, in DGC prepared semen in relation to pregnancy outcome was seen (Paper III). These findings support the two first SCSA–ART studies using prepared semen for SCSA. A limited number of patients, 24 and 34, respectively, were included in these studies (Larson *et al.,* 2000; Gandini *et al.,* 2004), why the present study may be considered the first SCSA study on this issue to be conclusive (Paper III). On the other hand, using the TUNEL assay, Borini et al. (Borini *et al.,* 2006) in ICSI patients found DFI>10% in DGC prepared semen to be discriminative for pregnancy. Also another study used washed semen samples and found no IUI pregnancy if DFI exceeded the level of 12% as measured by the TUNEL assay (Duran *et al.,* 2002).

Seen from a biological point of view, our finding is difficult to explain. Larson *et al.* (2000) suggested that elevated DFI in neat semen may reflect chromatin or other abnormalities within the entire sperm population interfering with the fertilising ability of the sperm, but not completely eliminated by DGC or swim-up. Although SCSA is a generalized test of sperm chromatin stability, it is unclear which type of sperm DNA breaks (single/double strand breaks) the method actually detects. It cannot be excluded that some unknown type of damage may be present in sperm without DNA denaturating during the SCSA procedure. These chromatin structure defects may thus remain in the DGC material without being reflected by DFI. Why such abnormalities, if related to sperm DNA breaks, cannot be measured by SCSA, remains to be elucidated. This could also explain the difference between

the SCSA and the TUNEL studies; the two methods may not completely reflect the same types of DNA damage (Makhlouf and Niederberger 2006).

In the present study (Paper III), despite the negative results in regard to predicting the outcome of ART, only in 1% of the samples, DFI was increased after preparation. With the exception of three samples, all samples having a DFI above 30% in neat semen, DFI decreased to below 30% after DGC. A mean (95% CI) decrease in DFI following DGC for all treatment groups of 15.1% (14.0–16.2%) was seen. The corresponding figures for HDS were 4.4% (4.0–4.9%). Scatter-plot diagrams demonstrated that for DGC samples, no DFI cut-off values could be set.

Several other authors have also demonstrated that the sperm preparation procedure can decrease the fraction of defective sperm initially present in the neat semen (Golan *et al.,* 1996; Larson *et al.,* 1999; Spano *et al.,* 1999; Donnelly *et al.,* 2000; Tomlinson *et al.,* 2001; Younglai *et al.,* 2001; Gandini *et al.,* 2004; McVicar *et al.,* 2004; Morrell *et al.,* 2004). On the other hand, there are also reports of unchanged or worse damage (Zini *et al.,* 1999; Zini *et al.,* 2000; Tomsu *et al.,* 2002). Also a majority of reports using other techniques for sperm chromatin integrity assessment failed to find any association between DFI in sperm population selected by DGC or swim-up and treatment outcome (Morris *et al.,* 2002; Benchaib *et al.,* 2003; Seli *et al.,* 2004; Huang *et al.,* 2005; Hammadeh *et al.,* 2006; Muriel *et al.,* 2006; Benchaib *et al.,* 2007). None of these studies were, however, based on such a large ART-population as our study.

The clinical recommendation resulting from Paper III is, that SCSA analysis should be performed on raw semen aliquots as also stated in the SCSA guidelines (Evenson *et al.,* 2002).

Intra-individual variation of the SCSA parameter DFI

In study IV, we found the mean coefficient of variation (CV) of DFI for 2-5 repeated SCSA measurements to be 29%, in other words in line with the intra-individual variation reported for other standard sperm parameters (Neuwinger *et al.,* 1990; Cooper *et al.,* 1992). Thirtyseven percent of patients having a DFI>30% in the first test had a DFI<30% in the second test. Twenty seven percent of those with DFI-values in the range of 20-30% in the first test had DFI above 30% in the second test (Paper IV).

Our findings are in contrast to previous reports regarding this issue (Evenson *et al.,* 1991; Evenson *et al.,* 2000; Zini *et al.,* 2001b; De Jonge *et al.,* 2004), finding the overall CV for DFI for repeated SCSA measurements to be 10-20%. However, most of these studies were based on rather limited numbers of both fertile and infertile men. In the study by Zini *et al.* (2001b), the men delivered two semen

samples, 2 to 6 weeks apart and in the study by Evenson *et al.* (2000), 45 men delivered semen samples monthly for 8 months. De Jonge *et al.* (2004) studied the variation of DNA fragmentation in relation to days of sexual abstinence in 11 men and found only a short (24-hour) abstinence period to have a negative influence on sperm chromatin quality. A more recent study, (Smit *et al.,* 2007) included two consecutive semen samples from 100 infertile men. This study confirmed that sperm DNA integrity has a lower biological variability than the classical semen parameters, which also was seen in previous reports. Smit *et al.* (2007) suggested that the sperm chromatin structure may be more influenced in patients with normal spermatogenesis, whereas in men with disturbed spermatogenesis, the chromatin structure may be so impaired already that the effect of unidentified factors leading to variability of sperm DNA fragmentation in time may not be as profound. In our study, CVs for DFI were similar in groups with normal and abnormal sperm concentration and motility; 28 and 31%, respectively.

A large number of studies have documented that factors like age, abstinence time, illness, fever, cancer treatment, smoking, medication and exposure to environmental hazards can alter sperm DNA integrity (Evenson *et al.,* 1991; Fossa *et al.,* 1997; Robbins *et al.,* 1997; Sun *et al.,* 1997; Rubes *et al.,* 1998; Potts *et al.,* 1999; Evenson *et al.,* 2000; Kobayashi *et al.,* 2001; Richthoff *et al.,* 2002; Saleh *et al.,* 2002a; Sanchez-Pena *et al.,* 2004; Stahl *et al.,* 2004; Rignell-Hydbom *et al.,* 2005; Rubes *et al.,* 2005; Sepaniak *et al.,* 2006; Stahl *et al.,* 2006; Sergerie *et al.,* 2007). While Saleh *et al.* found a 10-point decrease in ROS analysis scores, Sepaniak *et al.* found significantly higher DFI in smokers compared to non-smokers (32 versus 25.9%). In the study of Richtoff *et al.* DFI correlated positively with abstinence time (r=0.17). Fever was shown to increase DFI by 24% and 36%, 2 and 5 weeks after the fever, respectively. However, DFI returned to normal 10-12 weeks after the fever episode (Sergerie *et al.,* 2007).

In a recent study by Smit *et al.* (Smit *et al.,* 2007), patients who reported changes in medication or any significant illness or infection between different semen samples were excluded and all participants were instructed equally for abstinence time. Although we in our study also had information regarding most of the possible influencing factors, we did not use any of them as exclusion criteria. By not excluding any patients, we found that this would give the most correct picture of the true situation in a fertility clinic.

In order to identify men at risk of passing the 30% threshold, data were further divided into the following categories; i) DFI≤10%, ii) DFI=11%-20%. iii) DFI= 21-30% and iv) DFI>30%. The results showed that those having a DFI≤10% had only a 6% risk of having a DFI>30% in the following test. In the other groups; ii, iii and iv, the risk was 14%, 27% and 63%, respectively. Based on these data, one could recommend repeated SCSA testing in only those men who were not ICSI

candidates and having a DFI>20% since in this subgroup a switch of DFI to a higher level may have implications for the selection of the ART treatment.

New unpublished data indicates that the natural variability may not have a significant impact for how often SCSA needs to be performed. In the IUI follow-up study presented previously (pages 50/51), DFI was above 30% when the patient was included in the study, but we did not know if and how much DFI varied in periods during and between treatments. In another follow-up study (Giwercman *et al.,* unpublished data) including 142 unexplained sub-fertile men (no known female factor in infertility work up) and 135 proven fertile men as controls, the results showed that DFI was an independent predictor of infertility with OR of 2.5 (95%CI 1.0;6.1) for DFI between 10% and 20% and 8.4 (95% CI: 3.0; 23) for DFI≥20%.

Although DFI, as other sperm parameters, was found to be a subject of intra-individual variation (Paper IV), the results from these two unpublished works indicate that a single SCSA analysis is a strong predictor of infertility and that the intra-individual variation may not necessarily have a significant clinical impact. While waiting for new, larger studies, one should recommend the SCSA analysis to be performed shortly before the actual ART treatment.

Total fertilisation failure

Mutations in the PCI gene

One of the genes suggested to play a role in human male fertility is the Protein C inhibitor (PCI) gene (Espana *et al.,* 2007).

In men with total fertilisation failure in IVF trials, a novel mutation in the PCI gene was significantly more common compared to controls with normal IVF fertilisation rates (11 % vs. 0.0 %, p=0.02) (Paper V). This was a heterozygous A/G transition in position 1389 (rs206990), located in a non-coding part of the PCI gene and does therefore not result in any amino acid change. Two other mutations, not previously reported in the SNP database (www. ncbi.nih.gov) were also detected; a heterozygous G/C transition at position 1732 and a heterozygous A/G transition at position 1793. However, these were as frequent in patients as in the controls.

In total 15 different variants located in the PCI gene were identified. Except for the two novel nucleotide variants detected in a non-coding part of the PCI gene, all represented single nucleotide polymorphisms (SNPs) previously reported in the SNP database (www.ncbi.nih.gov). Two of the variants reported in the SNP database (rs2069975 and rs6117), were, however, not found in the present groups (Paper V).

A tendency for a more frequent distribution in the patient group compared to the controls was seen for a heterozygous T/C transition in exon 6 at position 1851 (rs9113) (p=0.07). Also other SNPs were detected in exon 6 and exon 3; however, these were as frequent in patients as in controls. For the remaining exons (1, 2, 4 and 5), no other deviations from the normal sequence were detected.

Even if the polymorphism in the PCI gene might be associated with fertilisation failure, it is important to point out that our findings can only partly explain this condition. The nature of fertilisation is highly complex and it is plausible to believe that the reasons for total fertilisation failure could be many e.g. technical, female as well as others of male origin.

A total of 15 different variants were compared and for one of them we found a statistically significant difference between cases and controls. Therefore, the problem of "mass significance" and hence a chance finding can not be excluded. The study should thus be seen as a pilot study. Future large-scale studies are needed to further elucidate the role of PCI in fertilisation failure. Consequently, at present, the clinical application of these findings is limited. It is likely that single gene mutations of the PCI gene account for a small proportion of male infertility and, therefore, the practicality of testing will be constrained by expenses and the availability of the testing techniques. However, if further large-scale studies can confirm and take these findings further, it may be possible and cost-effective to test idiopathic infertile couples for this gene defect prior to IVF. Moreover, in the future likely the use of cheap gene array testing techniques will evolve, which will enable us to screen all or most of the known single gene defects in one test.

Sperm DNA fragmentation

Study V also showed that total fertilisation failure after IVF could not be explained by elevated sperm DNA fragmentation.

Mean DFI and HDS values in couples in the fertilisation failure group were 19.8% (range 4.5-52%) and 9.6% (range 3-28.5%), respectively, and did not differ from those found in controls being 19.8% (range 6.6-67%) and 10.3% (range 4.1-18.9), respectively. The result was not unexpected, as we reported similar fertilisation rates in the high and low DFI groups in Study II, and the subjects in this case-control set up (Study V) represented a subset of patients included in Study II. These findings are in agreement with several other SCSA studies (Larson *et al.,* 2000; Larson-Cook *et al.,* 2003; Gandini *et al.,* 2004; Virro *et al.,* 2004; Li *et al.,* 2006).

In conclusion there is no a clear cut evidence of association between DFI or HDS and the ability to fertilise.

CONCLUDING REMARKS

With the present studies a significant progress in the clinical application of SCSA was made. Measures of genetic aberrations in terms of sperm DNA fragmentation can be used as markers of fertility, in order to decide which level of ART is needed; the simple IUI or more advanced methods, such as IVF or ICSI.

The main conclusions from the current studies are:

- DFI can be used as an independent predictor of success in couples undergoing IUI;
- A threshold value for DFI in regard to in vivo fertility can be set at 30%. When DFI exceeds 30%, ICSI should be the method of choice, also in cases where the traditional sperm parameters are normal;
- Fertilisation and cleavage stage embryo development (day 2 and day 3) is not influenced by DNA damage as measured by SCSA;
- HDS is not predictive for the outcome of ART;
- Although no statistically significant association between high DFI and early pregnancy loss was seen, one can not exclude the fact that DFI levels >60% are associated with higher risk of early pregnancy loss;
- Despite the fact that density gradient centrifugation effectively decreases DFI and HDS in semen, SCSA performed in semen prepared by density gradient centrifugation cannot predict the outcome of ART. SCSA analysis should therefore be performed in raw semen;
- The intra-individual variability of DFI is considerable. Men in couples undergoing ART, who are not candidates for ICSI, should be recommended SCSA prior and close to each ART treatment if DFI is above 20%;
- In a subgroup of cases total fertilisation failure after IVF might be explained by polymorphisms in the Protein C Inhibitor gene.

FUTURE PERSPECTIVES

The further challenges within the field are easy to identify. The first and maybe most important is to increase the focus on the role of paternal DNA damage in relation to ART. Until now, the fact that the sperm carries DNA and that the DNA can be of different quality interfering with men's fertility potential has been commonly overlooked in the diagnosis and treatment of male infertility. Using SCSA as a tool in our own clinical setting, a University clinic planning to run about 1500 ART cycles per year, together with the continuous spreading of our own as well as research data of others may contribute to a generally increased knowledge among those working with ART.

Based on the results presented in this thesis, it would be relevant to further penetrate the differences in success between ICSI and IVF in men with DFI above 30%. A study in which couples fulfil the traditional criteria for IVF and the men have a DFI above 30% should be randomised to either IVF or ICSI. This would clarify whether the latter method is superior in cases with high DFI.

In order to follow the "ROS-theory", suggested to play a role in the difference between IVF and ICSI, set up of ROS analysis in our clinic and using it in combination with SCSA to further study possible associations and mechanisms is of high priority. A positive correlation between DFI levels and the concentration of ROS in the seminal plasma has been demonstrated, however only in a limited patient material (Saleh *et al.,* 2003a). Analysis for measurements of ROS may be applicable to many other future research projects. One can be to use the analysis to identify men with ROS-induced DNA damage. This could contribute to the development of new treatment regimes for the use of antioxidants to minimize oxidative stress and sperm DNA damage as suggested previously (Greco *et al.,* 2005; Silver *et al.,* 2005; Menezo *et al.,* 2007). However new, larger scale studies are needed in order to identify potential groups that will benefit from such treatment. Moreover, dose finding studies in this kind of medication is needed.

The intra-individual variation of sperm DNA integrity in an ART setting should be further studied. Larger populations should be included where also the time factor and conditions known to interrupt with sperm DNA are taken into consideration. In a larger material and with possibility of additional analysis it may be possible to identify potential subgroups that are at particular increased risk of a significant intra-individual variation.

Some novel sperm selection systems to be used to select better spermatozoa for ICSI are suggested; a high-magnification optical system (Bartoov *et al.,* 2001), a electrophoretic system (Ainsworth *et al.,* 2007), a sperm hyaluronic acid (HA) binding assay (Huszar *et al.,* 2003) and a magnetic cell sorting system using

Annexin V microbeads (Said *et al.,* 2006). So far, the high-magnification optical system has demonstrated, in the most promising manner, the ability to isolate mature, viable sperm with unreacted acrosomal status, without damaging the specimen. However, the clinical applicability of these tests remains to be demonstrated in large scale studies.

Lastly, the findings presented in this thesis regarding the explanation of fertilisation failure in IVF should be followed by new, larger studies. In these studies quantification of PCI in the seminal plasma should be added to the analysis.

POPULAR SCIENTIFIC SUMMARY

In western countries, infertility is one of the most common disorders affecting approximately 15% of all couples. Traditionally, the diagnosis of male infertility has relied upon microscopic assessment of semen. The criteria for normality in regard to sperm concentration, motility and morphology are, however, claimed to be poorly standardized, subjective and not good indicators of fertility. In two thirds of the male infertility cases, no explanation of the reduced semen parameters can be found and the condition is, therefore, referred to as idiopathic infertility. With the advent of assisted reproduction (ART) and in particular intracytoplasmic sperm injection (ICSI) the importance of the sperm analysis tended to be minimal, based on the observation that ICSI bypasses natural fertilisation processes and that the method is independent of semen quality. However, over time, ICSI has become a subject to an ongoing debate regarding its indications and safety, especially in terms of transmission of genetic diseases to the offspring. Despite this and the fact that good criterias for the use of ICSI are lacking, its use is strongly increasing. In many clinics ICSI is actually the only fertilisation method used. In order to identify new parameters with a better potential to predict the chance of success or failure of natural conception as well as of ART, several parameters have been suggested, however, so far without any breakthrough.

Recent evidence suggests that a variety of genetic causes are likely to be associated with male infertility and can probably also explain many of the idiopathic cases of infertility. Sperm DNA damage, in terms of DNA fragmentation and/or changes in fertility related genes are suggested contributing to the problem and could therefore have the potential to be used as markers of fertility/infertility. The level of DNA damage as measured by one of the available analysing methods, the Sperm Chromatin Structure Assay (SCSA) is expressed as DNA fragmentation index (DFI) and high DNA stainability (HDS), the latter a measure of immature spermatozoa.

Infertile men are shown to have higher number of sperm with DNA damage compared to fertile men and two studies have shown that couples where the men have a high DFI (>30-40%) need longer time to obtain a pregnancy compared to couples where the men have a low DFI. Other studies have suggested a negative relation between sperm DNA damage and ART outcome. Additionally, a threshold levels for DFI in regard to fertility, measured in neat semen were suggested to be set at DFI 27% and it was questioned whether ART was able to compensate for poor sperm DNA quality. Moreover, it has been claimed that DFI is a more stable parameter than conventional semen parameters in the sense that the individual variation within a man should be less for DFI than for sperm concentration, motility and morphology - parameters normally used to diagnose male infertility.

The overall aim of the present studies was to identify genetic markers of fertility, in order to improve the diagnosis of male-dependent infertility. More precisely we aimed to evaluate whether any threshold values of DFI and HDS could be set in relation to pregnancy outcome of ART in neat- and density gradient centrifuged semen, and how DFI and HDS correlated to fertilisation rates and embryo development in *in vitro* fertilisation (IVF) and ICSI (Papers I-III). We also aimed to evaluate intra-individual variation of DFI in an ART population (Paper IV). Lastly, we aimed to study whether total fertilisation failure after IVF could be explained by DNA sequence changes in the PCI gene and also whether there was any association between fertilisation failure and SCSA parameters. A material of about 1000 ART treatment cycles are included in the studies.

In regard to fertilisation failure, the results of Paper V showed that a specific mutation in the PCI gene was significantly more common among male patients involved in IVF fertilisation failure compared to the control group who had normal fertilisation rates after IVF. Two other novel mutations were also detected. The results indicate that in a subgroup of IVF-patients total fertilisation failure may be caused by polymorphisms in the PCI gene (Paper V).

In contrast to previous reports the results from the present studies demonstrated that ART is able to compensate for poor sperm DNA quality, in particular if ICSI is chosen as fertilisation method. DFI can be used as a predictor of fertility and as a tool in choosing the most effective form of ART. We found that DFI can be used as an independent predictor of fertility in couples undergoing IUI. When DFI passed 30% the chance of obtaining a pregnancy by IUI was close to zero. On the other hand a DFI above 30% did not restrict pregnancy by ART. If the most invasive fertilisation method, ICSI, was chosen, the couple had the same chance to obtain a pregnancy as couples where the male partner had a low DFI. If DFI was above 30% ICSI was more effective than IVF. No impact of DFI or HDS was seen on fertilisation or embryo development. Althogh one could not exclude an increased risk that DFI levels >60% are associated with a higher risk of early pregnancy loss, no association between high DFI or HDS and early pregnancy loss was found for DFI levels below 60% (Paper II). Another important finding is that DFI should be measured in neat semen, not in processed. DFI in prepared semen did not predict pregnancy outcome of ART as raw semen did (Paper III). Moreover, DFI is not as stable as previous studies have claimed (Paper IV). Thus, DFI should be measured shortly before the actual ART treatment.

With these studies significant progress in identifying markers of male fertility in ART was made. Measures of genetic aberrations in terms of sperm DNA fragmentation can be used as markers of fertility, in order to decide which level of ART- treatment is necessary; the simple IUI or more advanced treatments, such as IVF or ICSI.

NORSK POPULÆRVITENSKAPELIG SAMMENDRAG

I hele den vestlige verden er ufrivillig barnløshet et hyppig forekommende problem. Omkring 15% av alle par opplever problemer med å oppnå ønsket graviditet, de er såkalt infertile eller subfertile. I løpet av de siste tre årtier har behandlingsmulighetene for ufrivillig barnløse par blitt vesentlig forbedret. Ved hjelp av såkalt assistert befruktning (ART) har man i dag flere ulike behandlingsmuligheter, alt fra enkel inseminasjonsbehandling (IUI) til in vitro fertilisering (IVF) (prøverørsbehandling) eller avansert mikroinjeksjonsbehandling (ICSI).

Under inseminasjonsbehandling gjennomgår kvinnen en enkel hormonstimulering hvorpå hun insemineres med mannens sædceller. Disse har på forhånd blitt renset opp med henblikk på å fjerne ikke funksjonsdyktige sædceller, samt andre celler fra prøven. Eventuell befruktning skjer således i kvinnens kropp. Ved IVF kreves en mer avansert hormonstimulering og eggene hentes ut fra eggstokkene for å bli befruktet i laboratoriet med mannens sædceller. Ca. 150 000 sædceller legges i en skål sammen med egget, og den spermie som befrukter egget finner selv veien inn i egget. To til fem dager senere legges 1-2 befruktete egg tilbake til livmoren. Ved ICSI følges samme behandlingsprinsipp, men befruktningen skiller seg fra IVF ved at en enkelt sædcelle føres inn i egget ved hjelp av en tynn nål.

Til tross for en enorm økning i kunnskapene på fertilitetsområdet, er utredningen av årsakene til barnløsheten stadig mangelfull, og kriteriene for hvem som skal ha hvilken behandling er uklare. Etter utredning står så mange som 25% av parene uten en diagnose, de er såkalt uforklarlig infertile. Så mange som to tredjedeler av mennene har ingen forklaring på hvorfor deres sædkvalitet er nedsatt. En manglende diagnose kan, på den ene side gi seg utslag i en begrenset effektivitet av behandlingene, og på den andre side i et mulig overforbruk av assistert befruktning, spesielt ICSI. Siden den første rapport om ICSI, som behandlig for nedsatt sædkvalitet ble publisert i 1992, har bruken av metoden økt markant, også ved normal sædkvalitet. Likevel har man ikke et klart bilde av om eventuelle mannlige kromosom- eller genfejl nedarves gjennom generationer eller om metoden i seg selv, på lengre sikt har uønskede konsekvenser. Enklere årsaksrelatert behandling gjøres i dag bare i mindre grad, men om flere par ble diagnostiserte, ville man trolig kunne behandle mange flere med enklere medisinsk behandling.

For mannens del, består utredningen av årsakene til barnløsheten tradisjonelt av en mikroskopisk bedømmelse av sædkvaliteten. Verdens Helseorganisasjon (WHO) har satt opp kriterier for hva som er normalt og unormalt i forhold til antall sædceller, bevegelighet og morfologi (utseende). Kriteriene har imidlertid vist seg å være usikre i forhold til å bedømme mannens evne til å oppnå graviditet med partneren. Mange menn med sædkvalitet under WHOs grense gir opphav til

graviditet, mens det på samme tid er vist at også menn med normal sædkvalitet har problemer med å gjøre sin partner gravid. Behovet for parametre som evner å gi et bedre mål på mannens fertilitetsevne er stort.

Det er mange årsaker til mannlig ufrivillig barnløshet; hormonelle, funksjonelle såvel som genetiske. Hos minst 15% av menn med nedsatt sædkvalitet mener man at det er genetiske årsaker som ligger bak barnløsheten. Kromosomfeil av ulik art er en hyppig årsak til mannlig infertilitet. En type kromosomfeil kan være at en viss andel av sædcellene har brudd i kromosomenes DNA-streng (sædcellens arvemateriale). De defekte kromosomene synes ikke ved vanlig mikroskopisk undersøkelse, men kan oppdages ved hjelp av såkalt flowcytometri analyse (i avhandlingen Sperm chromatin structure assay (SCSA)). Resultatet av SCSA analysen angis som DNA fragmenterings-index (DFI), et mål på hvor stor andel av sædcellene som har kromosombrudd, angittt i prosent og HDS (High DNA stainability), et uttrykk for andelen av umodne sædceller. SCSA metoden har så langt stort sett bare vært brukt innenfor veterinærmedisin og ikke i utredning av mannlig infertilitet. Studiene som inngår i denne avhandling omhandler anvendelsen av SCSA i behandling av ufrivillig barnløshet (Artikler I-V). I en av studiene (Artikkel V) utføres i tillegg mutasjonsundersøkelser av et bestemt gen (Protein C Inhibitor (PCI) gen) som i dyreforsök har vist seg å ha en avgjørende betydning for sædcellenes evne til å befrukte, spontant eller ved hjelp av IVF.

Materialet som er studert, er basert på sædprøver innsamlet fra ca 1000 menn i fertilitetsbehandling, alle samlet inn ved Fertilitetsklinikken, Sygehus Viborg, Skive, Danmark. Innen våre studier ble påbegynt hadde flere studier vist at ufrivillig barnløse menn hyppigere har sædceller med kromosombrudd, enn de som er fertile. Et par andre studier hadde også vist at par hvor mennene hadde høy andel av sædceller med kromosombrudd, brukte lengre tid på å oppnå graviditet spontant sammenlignet med par hvor mannen hadde sædceller med få kromosombrudd. En gruppe forskere i USA hadde også i noen mindre studier funnet at man ikke kunne oppnå graviditet ved assistert befruktning om DFI var over 27%. Andre mindre studier har vist at høy DFI og HDS har en negativ betydning for befruktning, utvikling av de befruktede egg (embryo) og for abortrisiko, men data var ikke konklusive. Disse funn var et viktig utgangspunkt for våre studier.

I avhandlingen presenteres resultater som blant annet viser at sjansen for å oppnå graviditet ved hjelp av inseminasjonsbehandling er nær null dersom DFI er høy (over 30%) (Artikkel I og II). Når ICSI velges som befruktningsmetode har imidlertid menn med høy grad av kromosombrudd i sædcellene (høy DFI) like gode sjanser for å oppnå graviditet med sin partner som menn hvor DFI er lav. Så mange som 20-25% av menn som søker fertilitetsbehandling har DFI over 30%. Til forskjell fra endel andre studier, men i samsvar med andre, viste artikkel II også at høy DFI og HDS ikke hadde negativ betydning for befruktning eller utvikling av

embryo (befruktete egg) (Artikkel II). Med hensyn til risiko for tidlig abort, fant vi ingen indikasjoner på at det var en sammenheng mellom DFI og abortrisiko, dog med en viss usikkerhet for de aller høyeste verdier (>60%) (Artikkel II). Resultatene fra artikkel IV, viste på samme måte at som de tradisjonelle sædkvalitets parametre, så varierer DFI fra prøve til prøve, dog likevel ikke i samme uttalte grad som for de tradisjonelle parametre. Optimalt bør SCSA gjøres i fertilitetsutredningen samt umiddelbart innen hver påbegynte fertilitetsbehandling.

For å oppnå en mest mulig ren sædprøve for befruktning i forbindelse med IUI, IVF og ICSI, gjør man en såkalt gradient sentrifugering (DGC). Sædprøven legges på en to konsentrasjons gradientløsning med høy tetthet og sentrifugeres. Gradienten filtrerer ut ubevegelige eller unormale sædceller og andre celletyper, og man står tilbake med en ren prøve for befruktningen. De fleste studier som omhandler emnet, har studert DFI og HDS i råsæd. Man kunne tenke seg at DFI i den prøve brukt for befruktningen best kunne forutsi utfallet av behandlingen, men artikkel III viser at det er DFI i råsæd som har denne egenskap.

Hos en viss andel av de par som gjennomgår IVF, mislykkes befruktningen, uten at man finner noen forklaring. Disse par kan i ny behandling hjelpes ved ICSI, hvor man mekanisk hjelper sædcellen inn i egget. Dette gir imidlertid ikke paret noen forklaring på den manglende befruktning og risikoen for at eventuelle genetiske feil nedarves vil være tilstede. Sannsynligvis ligger det mange mulige årsaker bak en mislykket befruktning, men forskningen på problemet har vært meget begrenset. I et enkelt dyreforsøk fant man at PCI-genet var en forutsetning for at sædcellen skulle kunne befrukte egget. I artikkel V ble DNA (arvemateriale) fra menn hos par som ikke hadde befrukning av eggene i sin første IVF behandling (Artikkel V) undersøkt. PCI-genet hos disse menn ble kartlagt og sammenlignet med en kontrollgruppe samt den normale variant av genet. Avhandlingen presenterer resultater som viser at for endel av de par som opplever manglende befruktning ved IVF, kan årsaken bero på forandringer (mutationer) i PCI-genet.

Samlet viser avhandlingen at man gjennom anvendelsen av nye genetiske markører for fertilitet i utredning og behandling av det ufrivillig barnløse par kan oppnå en mer målrettet, individuell og effektiv behandling. SCSA er et verdifullt redskab til å undersøke DNA-skade i sædceller hos ufrivillig barnløse menn. Innføring av SCSA analysen i utredning og behandling av ufrivillig barnløse par, bør kunne bety en bedre ressursutnyttelse. Fertilitesklinikkene kan gjøre bruk av DFI som en parameter til å utvelge den enkleste og best mulige behandlingsform for paret. For paret bør anvendelse av analysen bety en hurtigere vei til den rette behandling, en mindre belastende behandlingsprosess samt en større sjanse for å oppnå den ønskede graviditet. For de par som opplever eller risikerer manglende befruktning ved IVF, kan forklaringen ligge i forandringer i PCI-genet, men innen man kan anbefale en rutinemessig bruk av denne analysen, bør nye større studier utføres.

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