Sperm DNA Fragmentation - Infertility treatment, pregnancy, and the risk of congenital malformations

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Infertility treatment, pregnancy, and the risk of congenital malformations

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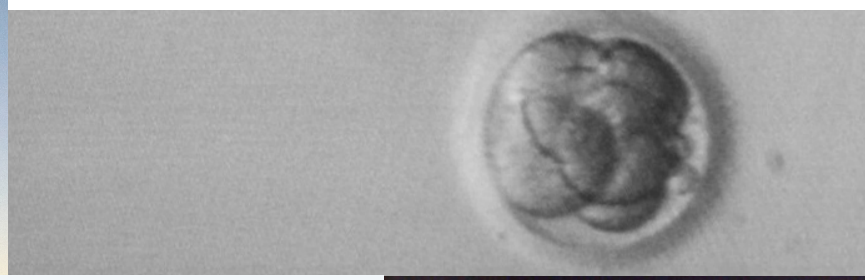

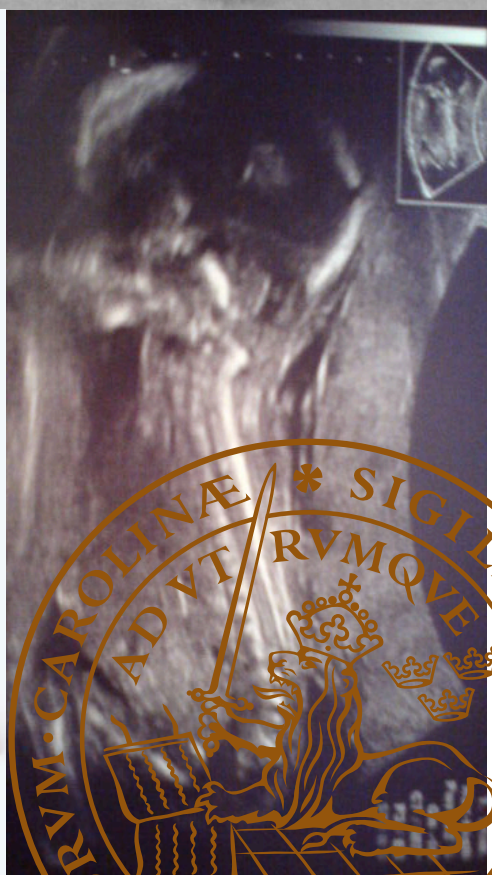

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AMELIE STENQVIST is currently doing her residency in Obstetrics and Gynecology at Skåne University Hospital, Malmö. The focus of this thesis is to investigate the impact of sperm DNA fragmentation on infertility treatment, pregnancy, adverse perinatal outcomes, and the risk of congenital malformations. Further, it evaluates the effect of antioxidant treatment in subfertile men with increased levels of sperm DNA fragmentation.

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Sperm DNA Fragmentation

Infertility treatment, pregnancy, and the risk of congenital malformations

Amelie Stenqvist

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 9th of December at 09.00 in the auditorium of the Department of Obstetrics and Gynaecology, Skåne University Hospital, Malmö, Jan Waldenströms gata 47.

Faculty opponent
Juha Tapanainen
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Abstract

Background: Elevated levels of sperm DNA fragmentation are associated with impaired male fertility and reduced chance of natural conception. With *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), fertilization and pregnancy can occur using spermatozoa with damaged DNA. There is limited knowledge of how this affects the pregnancy and the health of the offspring.
One of the causes of sperm DNA damage is oxidative stress. Antioxidants can decrease oxidative stress and are suggested as a treatment option to reduce sperm DNA fragmentation. There is a need for further randomized controlled studies investigating the effect of antioxidants on sperm DNA fragmentation.

Aims: This thesis aims to investigate the association between sperm DNA fragmentation and cumulative live birth rate, preeclampsia, preterm birth, low Apgar score, low birth weight, being small for gestational age, and congenital malformations. Further, it aims to evaluate the effect of combined antioxidant treatment on DFI in subfertile men with high sperm DFI.

Methods: Study I is a double-blind, randomized, placebo-controlled trial that evaluates the effect of combined antioxidant treatment on 77 subfertile men with increased levels of sperm DNA fragmentation. Studies II-IV are longitudinal cohort studies of couples undergoing assisted reproduction and their IVF- or ICSI-conceived children. These studies include data from medical records and, for studies III-IV, data from national medical registries. In all studies, sperm DNA fragmentation is measured by sperm chromatin structure assay (SCSA) as DNA fragmentation index (DFI).

Results: High sperm DFI was associated with a lower cumulative live birth rate and higher rates of preeclampsia in IVF cycles. No DFI-dependent differences in cumulative live birth rate or preeclampsia were seen in the ICSI group. When analyzing the whole cohort, both IVF and ICSI treatments included, there was an increased risk of preterm birth and congenital malformations in the high DFI group. Further, we found that antioxidant treatment did not affect DFI in infertile men with high DFI.

Key words: Infertility, sperm DNA fragmentation, sperm chromatin structure assay, in vitro fertilization, antioxidant treatment, cumulative live birth rate, preeclampsia, preterm birth, low birth weight, congenital malformations.

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When it comes to fertility, there are so many things that have to go right. In any one individual, there might be one major problem and two minor ones or no major ones and seven minor ones. Throw in another person's physiology, and it's complicated.

Robert Greene

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Abbreviations

ART	Assisted reproduction technique
BMI	Body mass index
CI	Confidence interval
CLBR	Cumulative live birth rate
CM	Congenital malformation
DFI	DNA fragmentation index
ET	Embryo transfer
FET	Frozen embryo transfer
ICD	International classification of diseases
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilization
LAS	Low Apgar score
LBW	Low birth weight
MBR	Medical birth register
NBHW	National board of health and welfare
NPR	National patient register
NRCA	National register of congenital anomalies
OR	Odds ratio
PE	Preeclampsia
PTB	Preterm birth
RCT	Randomized controlled trial
RMC	Reproductive Medicine Centre
ROS	Reactive oxygen species
SCSA	Sperm chromatin structure assay
SGA	Small for gestational age
SDF	Sperm DNA fragmentation
WHO	World Health Organization

Preface

When I started this PhD project, there were significant differences between fertility clinics regarding whether sperm DNA fragmentation (SDF) was investigated or not in infertility evaluations. Sperm DNA fragmentation analysis has been developed in recent decades and is not included in conventional semen analysis. It is known that analysis of SDF can explain some of the cases of infertility that are referred to as unexplained infertility since high levels of SDF can result in reduced fertility even if the conventional sperm analysis is normal.

There are advocates both for and against introducing the analysis more generally in infertility evaluations. All extra laboratory analyses cost money and time, and personnel must be trained to perform the analysis and assess the result. If SDF analysis is to become clinical practice, the benefits of the analysis need to exceed the costs.

In Malmö, where the work of this thesis has been carried out, it has been a clinical routine to analyze SDF for several years. Therefore, there was an existing extensive patient material and the possibility to include even more study participants prospectively in a large study with SDF in focus. We aimed to investigate the clinical importance of SDF not only in assisted reproduction techniques (ART) treatments but also in pregnancy and perinatal outcomes and in relation to the health of the offspring.

List of papers

The thesis is based on the following original publications and manuscripts, referred to in the text by their roman numerals.

I. **Stenqvist A**, Oleszczuk K, Leijonhufvud I & Giwercman A. Impact of antioxidant treatment on DNA fragmentation index: a double-blind placebo-controlled randomized trial. *Andrology* 2018; 6(6):811-816.

II. Malic Vončina S, **Stenqvist A**, Bungum M, Schyman T & Giwercman A. Sperm DNA fragmentation index and cumulative live birth rate in a cohort of 2,713 couples undergoing assisted reproduction treatment. *Fertility and Sterility* 2021; 116(6): 1483-1490.

III. **Stenqvist A**, Bungum M, Hansson S, Bisgaard Piborg A, Bogstad J, Englund A L, Grøndahl M-L & Giwercman A. Association between sperm DNA Fragmentation Index and the risk of preeclampsia as well as adverse perinatal outcomes following conception by IVF or ICSI. *Manuscript.*

IV. **Stenqvist A**, Bungum M, Bisgaard Piborg A, Bogstad J, Englund A L, Grøndahl M-L & Giwercman A. Association between sperm DNA Fragmentation Index and risk of congenital malformations in children conceived by IVF or ICSI. *Manuscript.*

Background

Infertility is an extensive problem affecting millions of people of reproductive age worldwide. About 1 of 7 couples are unable to conceive after one year of trying. Infertility can be caused by a variety of female and male factors, and in approximately half of the cases, a male factor contributes to the failure to conceive. For 15% of all couples, no cause is found in the infertility evaluation (1).

Much of the effort during an infertility evaluation is typically spent on the woman; who usually meets a physician that will take a careful medical history, perform ultrasonography, and measure hormone levels. Infertility evaluation of the male partner is often neglected (2). Men are, in most cases, expected only to provide a semen sample. Typically, a conventional semen analysis is performed, including semen volume, total sperm count, sperm concentration, and sperm motility and morphology assessment. This analysis has been shown to have a poor predictive value of fertility (3, 4). None of these parameters assesses the sperm's ultimate function – to provide intact and functioning male genetic material to the oocyte. In the last decades, several tests have been developed to assess the quality of this genetic material by measuring SDF.

In this introduction, I will first give a background of the cornerstone of this thesis: sperm DNA fragmentation. Different measurement techniques of SDF will be discussed as well as causes, clinical implications, and possible treatment strategies. Lastly, some outcomes of the studies will be introduced in the context of what is already known about the outcome in relation to paternal impact and ART.

Sperm DNA fragmentation

Like in somatic cells, the sperm cell has two types of DNA: nuclear and mitochondrial. Most of the sperm DNA is nuclear, located in the head of the sperm. It is only nuclear DNA that will be discussed in this thesis.

Sperm DNA is six times more tightly packed than the DNA in mitotic chromosomes of somatic cells (5). This is achieved by switching from histone- to protamine-based packaging, a process called protamination (6). The high level of compaction is believed to facilitate sperm motility and helps protect the DNA. Despite this, DNA damage frequently occurs in spermatozoa. One reason is that the sperm's capacity

to repair DNA fragmentation declines during spermatogenesis and mature sperm have no DNA repair mechanisms. However, the oocyte and early embryo are capable of DNA repair to a limited extent. This machinery is based on the maternal DNA repair capacity, and it seems that oocytes from women with normal ovarian reserve have better DNA repair potential than those from women with reduced ovarian reserve (7-9).

There are several types of DNA damage, such as base deletion or modification, DNA protein cross-linkage, and DNA fragmentation (10). DNA fragmentation refers to the separation of DNA strands, either single- or double-strand breaks.

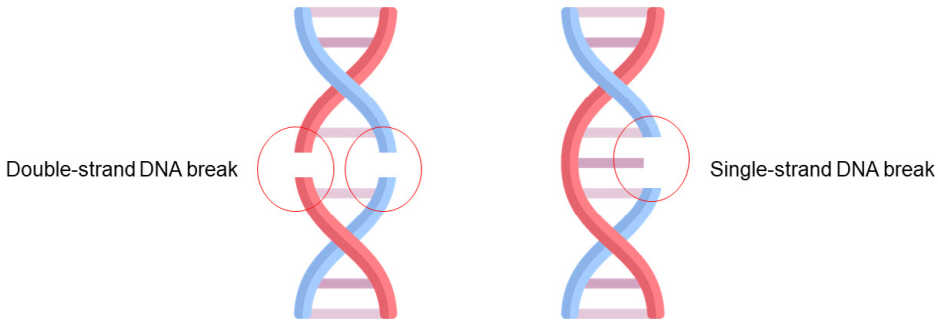

Figure 1. Schematic view of double and single DNA strand breaks

Measurement of sperm DNA fragmentation

A number of tests have been developed to analyze SDF. The tests differ in several ways. Some measure DNA breaks directly, and some require an initial denaturation to detect them. There is a difference in which kind of DNA breaks different tests detect, single- and double-stranded DNA breaks or only double-stranded. A selection of the available measurement techniques will be presented below, with a comparison of the different tests.

Sperm Chromatin Structure Assay (SCSA)

The technique used in sperm chromatin structure assay (SCSA) was first described by Evenson *et al.* in 1980 (11). The test measures sperm DNA susceptibility to denaturation, which is strongly correlated with DNA fragmentation (12). Initially, the sperm sample is diluted in a buffer to a concentration of 1-2 x 10^6/mL. Consequently, this technique cannot be used in case of severe oligospermia. The sperm sample is exposed to acid, which opens the DNA strands at sites of DNA breaks. Immediately after, the sample is stained with the fluorescent dye acridine orange. About 5000 spermatozoa are measured by flow cytometry, using computer-

assisted analysis. Intact sperm emits green fluorescence, and sperm with fragmented DNA emits red fluorescence. The red to red + green fluorescence ratio reflects the percentage of sperm with fragmented DNA, referred to as the DNA fragmentation index (DFI). Both single- and double-stranded DNA breaks are detected by SCSA. To compare data with others and ensure low intra-laboratory variability, a standardized protocol must be used (13).

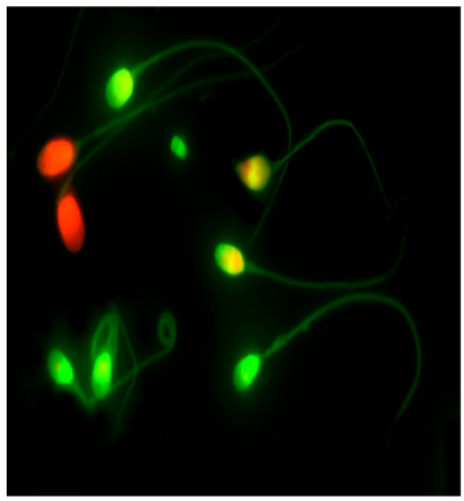

Figure 2. Acridine orange staining of sperm chromatin. Spermatozoa with intact double-stranded DNA emit green fluorescence. Spermatozoa with fragmented DNA emit yellow to red fluorescence.

Acridine Orange Test (AOT)

The acridine orange test (AOT) is based on the same technique as SCSA, but the assessment is made subjectively in a fluorescence microscope instead of by flow cytometry and subsequent computer-based analysis. It is cheaper than SCSA since a flow cytometer is not needed but is limited by the subjective assessment and evaluation of a smaller number of spermatozoa. Another disadvantage of the technique is that the fluorescence emissions rapidly fade, making the assessment challenging.

Single Cell Gel Electrophoresis (COMET)

In single cell gel electrophoresis (COMET) (14), sperm are embedded into agar on a microscope slide. Next, a lysis process is performed to reveal the DNA, which is then incubated in a neutral or alkaline electrophoresis solution. Electrophoresis is conducted where broken DNA strands migrate towards the anode, resulting in a

"comet tail" observed in fluorescence microscopy. The assay is based on the evaluation of this image. The assessment is made either subjectively or by a computer-assisted analysis program. The COMET assay has been applied in several different protocols. Neutral COMET detects double-stranded DNA breaks, and the alkaline version detects both single- and double-stranded DNA breaks, indistinctively. There are also COMET assays that can differentiate between single- and double-strand DNA breaks in the same sperm cell (15).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (16) is based on the enzymatic incorporation of deoxyuridine triphosphate (dUTP) into single- and double-stranded DNA fragments. The enzymes label the free ends of DNA. Sperm is assessed either subjectively in a fluorescence microscope or by flow cytometry. When evaluated in a microscope, only a limited number of spermatozoa is needed, which means this technique can be used in cases with severe oligospermia.

Sperm Chromatin Dispersion Test (SCD)

This test is also referred to as the Halo test (17). Sperm samples undergo acid denaturation to reveal fragmented DNA. Next, the sperm sample is stained, and spermatozoa with non-fragmented DNA produce a "halo" that can be evaluated in a fluorescence microscope. The recommended sperm concentration is 1-3 x 10^6 /mL for analysis, but samples with lower concentrations can also be used.

Comparison between the different SDF measurement techniques

Using a flow cytometer as in SCSA and the flow cytometric version of TUNEL is more objective than the microscopic evaluation made in SCD, AOT, COMET, and conventional TUNEL. Flow cytometer and computer-based assessment minimize the inter-observer variability but are, on the other hand, more expensive. In SCSA and the flow cytometric versions of TUNEL, about 5000 spermatozoa are measured in contrast to the microscopic assessment in SCD, AOT, COMET, and conventional TUNEL, where only 50-500 spermatozoa are evaluated. However, a disadvantage of the flow cytometric tests is that they require a larger number of spermatozoa (in SCSA, a sperm concentration above 1x10^6/mL is preferable), which means these methods cannot be used in cases of severe oligospermia.

There have been concerns about considerable inter-laboratory variability in SDF assessment (18). In SCSA, this problem is minimized by using a standardized protocol that has been fixed in the last 30 years (13).

Causes of sperm DNA fragmentation

Several factors, such as lifestyle, aging, diseases, and exposure to environmental toxicants and pollutants, have been associated with SDF (19). DNA fragmentation can occur at any stage during spermatogenesis, and sperm with fragmented DNA is found in both testicular, epididymal, and ejaculated sperm. At the molecular level, DNA fragmentation is mainly induced by three mechanisms: failure during chromatin compaction, abortive apoptosis, and oxidative stress (10, 19). Failure during chromatin compaction and abortive apoptosis is often referred to as testicular mechanisms, as this occurs within the testis, and oxidative stress as post-testicular, as this occurs throughout the male genital tract (20).

During chromatin compaction, DNA breaks are necessary for the process where histones are replaced with protamines. During this step, topoisomerase II activates, which repairs the DNA breaks. If this fails, and these breaks remain unrepaired, it can result in persistent DNA breaks in the ejaculated sperm (21). In addition, impaired chromatin compaction also makes the sperm more susceptible to reactive oxygen species (ROS) attack (19).

Apoptosis, or programmed cell death, is a normal physiological process. In the testes, germ cell apoptosis is crucial to maintain the right balance between germ and Sertoli cells and to control sperm production. Defective germ cells can escape the normal apoptosis process, which results in spermatozoa with apoptotic signs, including DNA fragmentation (18).

The most common cause of SDF is oxidative stress (22). It appears when there is an imbalance between the production and accumulation of ROS and the antioxidant system. Reactive oxygen species have, for a long time, been implicated as a possible cause of male subfertility. Somewhere between 30-80% of subfertile men have elevated levels of ROS (23). Spermatozoa are vulnerable to ROS since the plasma membrane consists of significant levels of polyunsaturated fatty acids, which are targets for ROS, and the cytoplasm only contains a limited amount of antioxidant factors (10). Lipid peroxidation causes increased membrane permeability, which can lead to DNA damage. Elevated levels of ROS are associated with impairment not only in conventional semen parameters but also in SDF (23). Reactive oxygen species may come from exogenous sources like smoking, alcohol, diet, radiation, and environmental toxicants and from endogenous sources such as varicocele and leukocytes.

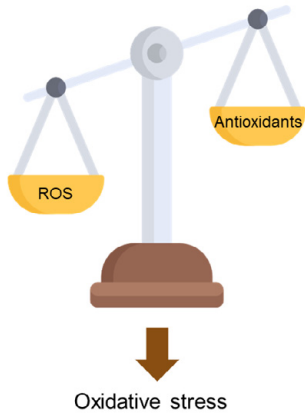

Figure 3. An imbalance between antioxidants and ROS results in oxidative stress.

Varicocele leads to increased ROS production in the testis, and about 50% of varicocele patients have elevated levels of SDF (24, 25). Cancer (26, 27) and diabetes (28-30) are other diseases that also have been associated with increased SDF, and, for diabetes, higher levels of ROS have been found in the ejaculate compared to healthy controls. Infections of the male reproductive tracts are associated with increased levels of oxidative stress and DNA fragmentation (31, 32). This is partly explained by the increased number of seminal leukocytes that contribute to ROS production and increased scrotal temperature caused by fever (20). Not only diseases but also treatments can have a detrimental effect on sperm DNA integrity, and there are reports of elevated levels of SDF as a consequence of chemo- and radiotherapy (33).

A number of lifestyle factors seem to have a negative effect on sperm DNA integrity. Alcohol (34), smoking (35), and cannabis use (36) have been reported to increase SDF. A healthy diet is associated with lower levels of SDF (37, 38). However, it is not clear whether obesity is a risk factor for SDF since the data are conflicting (39). Other factors associated with a negative effect on sperm DNA integrity are environmental and occupational exposure such as radiation (40-42), pesticides (43, 44), and air pollutants (45-47).

The association between age and SDF is reported in many studies (48-50), and recently an extensive study of over 25 000 men attending infertility clinics confirmed the association between age and SDF. The effect of male age on sperm integrity was particularly evident in men older than 41 years (51). It is speculated whether this finding is due to a decline in the DNA repair system of spermatids with age and accumulated exposure to environmental toxicants over time (19).

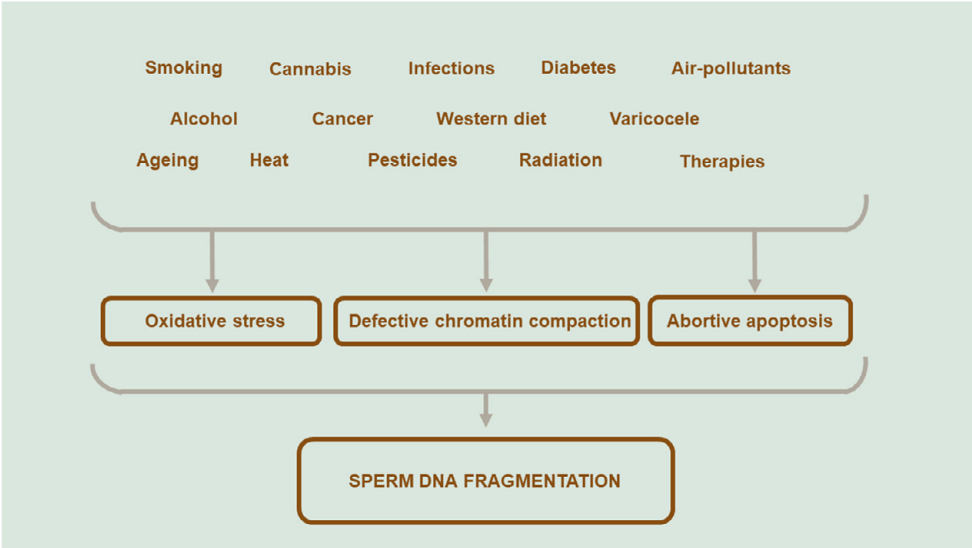

Figure 4. Different factors can induce SDF through mainly three mechanisms.

Treatment options for high sperm DNA fragmentation

It is preferable to offer subfertile men with elevated SDF to undergo a reproductive evaluation to see if there are any underlying causes that are treatable (10, 52). Treatment strategies depend on the suspected cause, and in this section, some treatment options will be presented.

Antioxidants

Since oxidative stress occurs when there is an imbalance between ROS and antioxidants, it is appealing to try to add more antioxidants to reach equilibrium and reduce oxidative stress. At the beginning of this PhD project, there was, to the best of my knowledge, only one randomized controlled trial (RCT) published investigating the effect of antioxidants on SDF (53). In that trial, 64 men with elevated SDF from couples with unexplained infertility were randomized to either Vitamin C and E treatment or placebo for two months. The treatment group had significantly reduced SDF values at the end of the trial compared to pre-treatment values. Since then, a few more RCTs have been published, with conflicting results (54-57). A recent Cochrane review (58) concluded that there is low certainty evidence that antioxidant supplementation in subfertile men improves clinical pregnancy and live birth rates. The authors further argued that more randomized, placebo-controlled trials are required to elucidate the role of antioxidant supplementation among subfertile men. A problem in the comparison between different antioxidant trials is that there is a large diversity in which antioxidants, dosages, and duration of treatment that have been applied. Furthermore, there is a

large diversity in the study populations, and most of the trials are not placebo-controlled but are a comparison between different antioxidants and dosages.

Concerns have been raised regarding treatment with high dosages of antioxidants and their risk of leading to a paradoxical effect and impairing sperm function instead of improving (20, 23). This is due to the knowledge that a certain level of ROS is needed for several physiological events during sperm maturation and the fertilization process. However, antioxidant supplementation is easily available, can be bought without a prescription, and there is explicit marketing by antioxidant supplement companies claiming to improve sperm parameters and fertility. All in all, this means that many infertile couples use various antioxidant supplements, even without a recommendation from a healthcare provider and despite the lack of evidence that these commercial antioxidant supplements have the effect that the advertisements say.

In theory, treating oxidative stress-triggered SDF with antioxidants is appealing. However, more well-designed RCTs are needed to determine who would benefit from this treatment and which antioxidants and dosages are the most appropriate.

Other treatment strategies

Varicocele is common in infertile men and is, as previously described, associated with oxidative stress and increased SDF (25). A recent meta-analysis concluded that DFI in varicocele patients decreased significantly after varicocelectomy (59). The summary evidence has resulted in several review articles claiming that varicocelectomy should be considered in subfertile men with increased SDF (10, 20).

The level of SDF is higher in ejaculated sperm than in testicular sperm (60). To get past the potential damage that occurs during passage through the reproductive tract, surgically retrieved spermatozoa are an option. Guidelines recently published by the European association of urology suggest that couples with high SDF could benefit from testicular sperm extraction and ICSI (61).

In contrast to varicocelectomy and retrievement of testicular sperm, which are surgical procedures that can never be entirely risk-free, lifestyle-related changes such as a healthy diet, reducing alcohol intake, and quitting smoking are risk-free. These lifestyle factors are reported to be associated with SDF, but it is not yet demonstrated that an intervention could reduce SDF.

It is known that SDF increases with abstinence time (62-64), and a short abstinence time is also shown to improve pregnancy rates following ICSI (62). Therefore, a short abstinence time can be used as another risk-free way of reducing SDF (20).

An assessment regarding genital tract infection should be performed during an infertility evaluation. Infection-induced SDF has been shown to respond well to antibiotics, with decreased SDF levels as a result (31).

Clinical implications of sperm DNA fragmentation

Several studies have reported higher levels of SDF in subfertile men compared to those proven fertile (65, 66). A review and meta-analysis by Santi *et al*. suggested a threshold value of SDF 20% to best discriminate between subfertile and confirmed and presumed fertile men (67). However, it is worth noticing that infertility is a couple's problem, and using only a single factor to predict fertility works quite poorly since it is an interaction between many factors and also the female partners' fertility potential (10). Nevertheless, SDF is associated with several adverse reproductive outcomes, which will be further discussed in this section.

Level of SDF influence both natural conception and the outcome of infertility treatments. In a study of first pregnancy planners from the general population, fecundity reduced rapidly at SDF > 20% (68). The chance of pregnancy following intrauterine insemination (IUI) is also reduced as a consequence of sperm DNA damage, with pregnancy rates declining at SDF > 20% and markedly reduced above 30% (69). A review and meta-analysis by Chen *et al*. concluded that high SDF was associated with lower pregnancy and delivery rates after IUI (70). Regarding *in vitro* fertilization, two meta-analyses have concluded that high SDF is associated with decreased pregnancy rates after IVF but not ICSI (71, 72). On the contrary, a more recent meta-analysis by Simon *et al.* showed that SDF negatively affected pregnancy rates both in IVF and ICSI (73). However, unlike natural conception and IUI, where the chance of pregnancy approaches zero if SDF > 30%, fertilization and pregnancy can occur with IVF and ICSI despite high levels of DNA damage. A review by Esteves *et al.* remarks that the adverse effects of SDF seem to be less evident in ICSI compared to IVF (10), but the data are conflicting. It is still a matter of debate whether ICSI is preferable in cases of high SDF or if conventional IVF could be used with the same success rate.

A meta-analysis by Robinson *et al*., based on 16 studies, showed an increased risk of miscarriage with elevated SDF. Not all included studies report associations between miscarriage and SDF, but among those that do, an increased risk can be seen both after natural conception and infertility treatments (74). Recurrent pregnancy loss has been shown to be more common when the male partner has elevated levels of sperm DNA damage, and several review articles suggest that affected couples should be recommended to analyze SDF (10, 18, 75). A meta-analysis showed that SDF was higher in couples experiencing recurrent pregnancy loss, as well when analyzing all studies, regardless of SDF measurement technique, as when analyzing SCSA studies separately (76). Since SDF is associated with poor embryo development (77, 78), it has been speculated whether this could be one of the mechanisms behind the increased level of recurrent pregnancy losses within couples with high SDF (75).

The effect of SDF on live birth rates (LBR) is less studied than the effect on pregnancy rates. In a meta-analysis including six ART studies, LBR was higher in

couples with low SDF. There was no difference in LBR between low and high SDF when ICSI was used as the fertilization method (79).

The effect of SDF on perinatal outcomes is even less studied. A study with 131 singleton pregnancies showed no significant differences in birthweight or gestational length between different SDF groups (80). In another study of 713 ICSI delivery cycles, no differences were seen in neonatal outcomes, including prematurity and birth weight (81). Birth defects were also reported, but with only seven cases of congenital malformations in the whole cohort, it is difficult to draw any conclusions.

Preeclampsia and adverse perinatal outcomes

Preeclampsia (PE) and adverse perinatal outcomes such as premature birth (PTB), low birth weight (LBW), and being small for gestational age (SGA) are more common in ART pregnancies and ART-conceived children compared to naturally conceived. A partial explanation is the increased proportion of multiple pregnancies among ART-conceived. Still, an elevated risk of PE and adverse perinatal outcomes remains even when analyzing only singleton births (82, 83).

Preeclampsia is a complex pregnancy-related disorder that affects 3-7% of pregnant women (84). The previous definition of PE was based on the development of hypertension and proteinuria. According to the current definition, PE is diagnosed in the case of high blood pressure after 20 weeks of gestation and at least one of the following findings: proteinuria, acute kidney injury, liver dysfunction, thrombocytopenia or hemolysis, neurological features, or intrauterine fetal growth reduction (85). The disease is associated with increased fetal and maternal mortality and morbidity, including severe adverse outcomes such as impaired fetal growth and preterm birth. It is estimated that globally preeclampsia is responsible for over 70 000 maternal deaths and 500 000 fetal and neonatal deaths every year (85), the vast majority in developing countries. The disease can be fatal even in the western world, and PE is the most common cause of maternal mortality in Sweden (86).

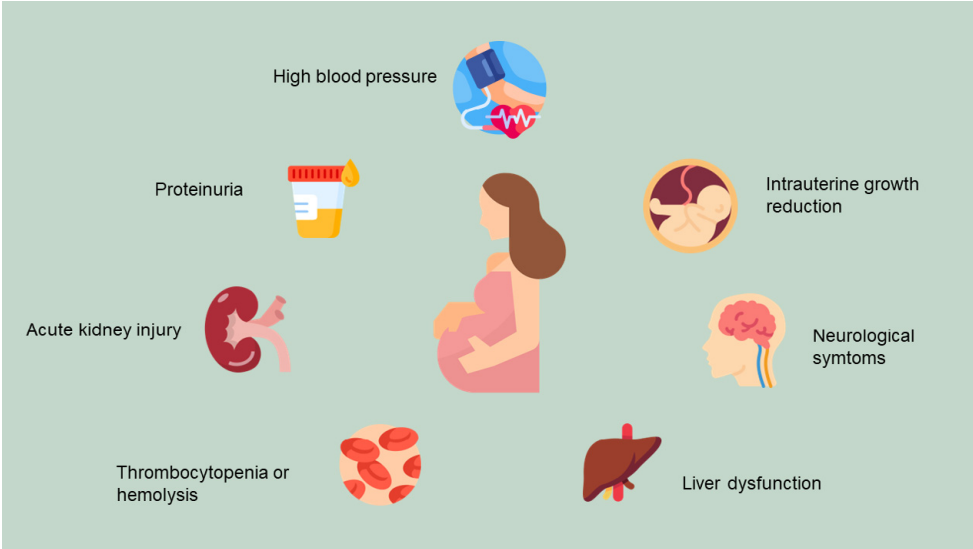

Figure 5. Clinical manifestations of preeclampsia

There is growing evidence suggesting that paternal factors contribute to the onset of PE. In 1981, a case report was published in Lancet, describing how a man lost his wife due to severe early-onset PE (87). He re-married, and his second wife also developed severe preeclampsia and died. The authors are suggesting the existence of a "father factor". Since then, more studies have been performed to elucidate the paternal impact on the development of the disease. If a woman experiences PE in her first pregnancy, partner change decreases the risk of getting PE in a subsequent pregnancy (88). On the contrary, partner change can have a predisposing role if a woman did not have PE during her first pregnancy (89). Further, a woman pregnant with a man who previously had a partner with PE has an increased risk of developing PE herself (90). These findings have led to the theory of the "dangerous father", but the underlying mechanism is not known (91).

It is reported to be an increased risk of PE in women with a short duration of sexual relationship (92) and the use of barrier contraceptive methods (93). Researchers have hypothesized that exposure to antigens in seminal fluid induces maternal tolerance to paternal antigens, which acts protective against PE (91). However, some studies have contradictory results showing no association between seminal exposure and PE risk (94).

Not only PE but also perinatal outcomes have been shown to be influenced by paternal factors (95). A large population-based cohort study including >40 000 000 live births reported advanced paternal age being associated with increased risk of PTB, LBW, and low Apgar score (LAS) (96). However, findings are conflicting, and in a systematic review from the same year, the authors concluded that there

might be little or no difference in risk of PTB, SGA, or LBW between younger and older fathers (95). In the same article, a meta-analysis showed that paternal smoking implied a statistically significant increased risk of SGA. The authors speculated whether smoking-induced sperm DNA damage could be a possible explanation for such association, but evidence for this pathogenesis is lacking (95).

Congenital malformations

It is estimated by the World Health Organization (WHO) that approximately 6% of all children are born with a congenital malformation (CM) (97). The reported incidence of CM differs in studies, partly because of differences in surveillance and reporting between countries. CMs vary substantially in severity. Hence, CM is often referred to as minor or major. The European Registration of Congenital Malformations and Twins (EUROCAT) is a network of population-based registries for the epidemiological surveillance of congenital malformations. According to their data, 2.5% of all European children are born with a major malformation that has a significant medical, social, or cosmetic consequence for the affected person (98).

Congenital malformations are more common in ART-conceived children (99). It is believed that the increased malformation rate is mainly due to the factors underlying infertility and not the ART treatment *per se*. This conclusion is based on the finding that subfertile couples who conceive naturally have a higher risk of CM than fertile couples, and the risk increase with increasing time to pregnancy (100). However, the mechanisms behind the association between subfertility, ART, and CM are unknown (101).

Ever since it was shown that DNA-damaged spermatozoa could fertilize oocytes (102), there has been a concern about how this would affect the health of the children. In an animal study with mice, fertilization with DNA-fragmented sperm led to offspring with increased anxiety, lack of habituation patterns, memory deficit, premature aging symptoms, and tumors (103). Paternal smoking is associated with an increased risk of CM (104), and it is speculated that sperm DNA damage is one of the mechanisms behind this finding. In summary, there is almost a complete lack of knowledge about the effect of SDF on the health of the children.

Rationale

The use of ART is increasing. In 2020, 4676 IVF- and ICSI-conceived children were born in Sweden (105). Approximately 20-30% of these children have fathers with elevated levels of SDF (106), many with such a high proportion of DNA-damaged spermatozoa that natural conception would have been unlikely. With today's effective ART treatments, this can be overcome; fertilization and pregnancy can occur with the use of spermatozoa with DNA damage (102). There is very little knowledge of how this affects perinatal outcomes and the children's health. It is well described that ART-conceived children have an increased risk of CM (99), but the underlying pathophysiological mechanisms are unclear. There has been a concern that SDF could increase the CM risk, but no study investigating this aspect has yet been published. Furthermore, ART-pregnant women are at increased risk of PE and adverse perinatal outcomes such as PTB and intrauterine growth restriction (82). It is important to find who within this heterogeneous group of ART couples is at higher risk for these adverse events in order to gain a better understanding of the underlying mechanisms, but also to better prevent adverse outcomes, and to be able to treat the underlying cause. One reason for the lack of studies investigating these questions is probably that it requires a large study population, a fertility center where it is routine to analyze SDF, and access to medical registries for follow-up of the children. Sweden is one of few countries where it is possible to access comprehensive and high-quality data from national medical registries, making these kinds of studies well suited to be performed here.

Assisted reproduction technique treatments are resource-consuming, both for the affected couple and society. Further, ART treatments are not risk-free for the woman. Everything considered it is essential to make the treatments as effective as possible in terms of LBR. Some evidence indicates that in cases with high SDF, ICSI is more efficient than IVF (71, 72). However, including SDF analysis in the decision-making regarding treatment type is not yet a clinical routine. In the vast majority of fertility clinics, no SDF analysis is done. Choosing between IVF and ICSI in the first treatment cycle is most often based on standard semen parameters and the yield of motile spermatozoa following gradient centrifugation or swim-up. In the following treatment cycles, the choice of method is also dependent on the outcome of the first cycle. It is, therefore, important to investigate how the choice of first ART treatment affects the cumulative live birth rate (CLBR) in couples with high SDF. Finding treatment strategies that are more effective for this patient group could lead to reduced financial costs, reduced medical risks for the women, and ultimately more children born.

Aims

The overall aim of this thesis is to investigate the clinical impact of SDF, measured by SCSA as DFI, throughout the journey for infertile couples – from the choice of first ART treatment to adverse pregnancy and perinatal outcomes and the health of the offspring.

The specific aims are:

I. To evaluate the effect of combined antioxidant treatment on DFI in subfertile men with high sperm DFI.

II. To study how the choice of first ART treatment type (IVF or ICSI) affects the cumulative live birth rate in couples with high sperm DFI.

III. To study the association between sperm DFI and the risk of preeclampsia, preterm birth, low birth weight, low Apgar score, and being small for gestational age after IVF and ICSI.

IV. To study the association between sperm DFI and the risk of congenital malformation in children conceived by IVF or ICSI.

Subjects and methods

Study design and overview

This thesis is based on four studies performed at the Reproductive Medicine Center (RMC) at Skåne University Hospital, Malmö. Study I is a double-blind, randomized, placebo-controlled trial, and studies II-IV are longitudinal cohort studies. The studies are summarized in Table 1.

Table 1. Overview of the studies included in the thesis
Note: DFI = DNA fragmentation index. RCT = randomized controlled trial. ART = assisted reproductive techniques. CLBR = cumulative live birth rate. PE = preeclampsia. PTB = preterm birth. LBW = low birth weight. SGA = small for gestational age. LAS = low Apgar score. CM = congenital malformations.

	STUDY I	STUDY II	STUDY III	STUDY IV
Brief title	Effect of antioxidant treatment on DFI	DFI and cumulative live birth rate	DFI, preeclampsia and adverse perinatal outcomes	DFI and congenital malformations
Study design	RCT	Longitudinal cohort study	Longitudinal cohort study	Longitudinal cohort study
Participants	77 subfertile men with high DFI	2713 infertile couples	1594 couples and their 1660 ART-conceived children	1772 ART-conceived children
Data sources	Physical examination, semen- and blood analysis	Medical records and national register data	Medical records and national register data	Medical records and national register data
Exposure	Antioxidants or placebo	DFI	DFI	DFI
Primary outcome(s)	DFI	CLBR	PE, PTB, LBW, SGA, LAS	CM

Subjects

This thesis is based on two cohorts: cohort A, used in study I, and cohort B, used in study II-IV.

Cohort A

Men from infertile couples who had visited RMC for infertility evaluation and had DFI ≥ 25% were assessed for eligibility. Attempts were made to contact these men by telephone. Information about the study was given, and questions were asked to further evaluate whether the man met the inclusion criteria. To be included in the study cohort, the man should be between 18-50 years old, a non-smoker who had never used anabolic steroids, and had not taken antihypertensive drugs, hormones, statins, psychotropic drugs, oral cortisone, or antioxidant supplementation during the last six months. If the man met these criteria and agreed to participate in the study, he was invited to a screening visit. Hight and length were measured, and blood and semen samples were collected. Men with BMI ≤ 30, normal sex hormone levels, and DFI ≥ 25% in this repeated semen sample were included after signing an informed consent form.

In total, 613 men were contacted by telephone. One hundred sixty men were interested in participating in the study and met the parts of the inclusion criteria that could be verified in the first telephone conversation. These men all came to a screening visit. Seventy-nine men fulfilled all inclusion criteria and were randomized, but soon after, two men announced that they wanted to discontinue due to their inability to stick to the schedule and come back to deliver semen samples after three and six months of treatment. Consequently, 77 men were included in the analyses; 37 were randomized to antioxidant treatment and 40 to placebo.

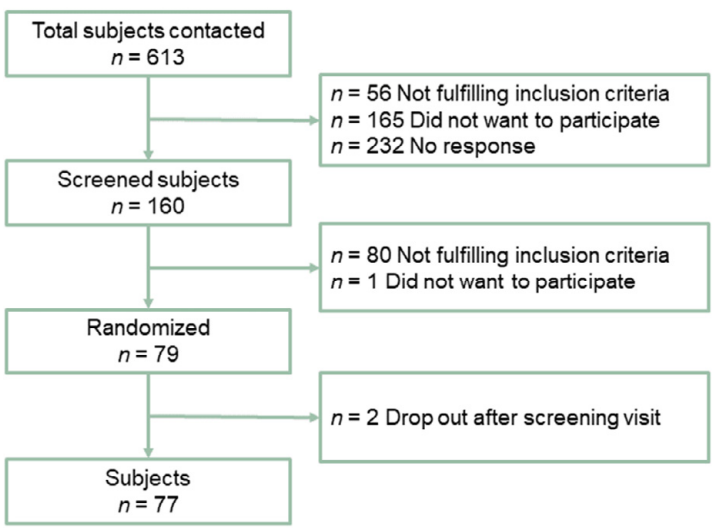

Figure 6. Flow chart describing the inclusion process of cohort A.

Cohort B

This cohort comprises infertile couples who have undergone IVF or ICSI at RMC, Malmö.

Couples were included, either retrospectively or prospectively. All couples who had undergone IVF or ICSI treatment between 2007-2018 and had at least one DFI value assessed were asked about participation in the study. In the retrospective inclusion, couples were contacted by letter with information about the research project and offered an opt-out if they did not want their data to be included. The couples could also be included during a visit to RMC before starting ART treatment. In the prospective inclusion, the couples were given verbal and written information about the study. If they agreed to participate, both partners signed an informed consent.

Since the clinic provides public healthcare, the patient fee is low and only covers a small percentage of the costs. However, the couples need to fulfill some criteria to undergo treatment:

- Failure to conceive after at least 12 months of unprotected intercourse or known severely impaired male or female fertility
- Both partners being non-smokers
- Females younger than 40 years
- Males younger than 56 years
- Female BMI between 18-30 kg/m^2, or 10% weight loss if BMI >30 and <35kg/m^2.
- No common child. Exceptions are made if a couple has a child after a successful treatment and there are remaining frozen embryos. Those embryos can be transferred, but no new fresh treatments are made.

Throughout the study period, it has been clinical routine to analyze DFI on all sperm samples used for ART treatments. In order to measure DFI by SCSA, a sperm concentration of > 1 x 10^6/mL is required. This means that couples, where the male partner had lower sperm concentration than this threshold, could not be a part of this cohort. Further, couples using donated gametes were excluded.

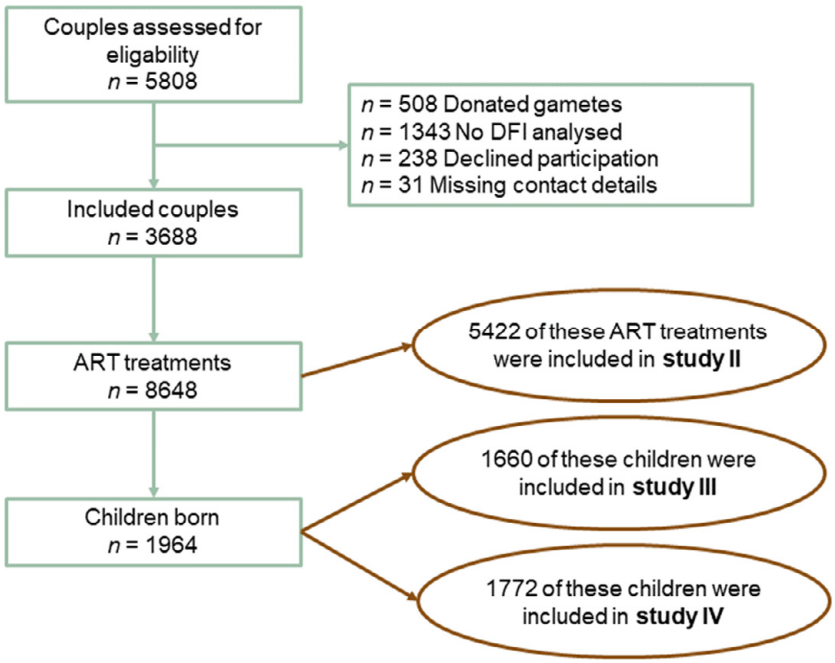

Figure 7. Flow chart describing the inclusion of cohort B.

In study II, 5244 of the ART treatments of cohort B were included in the final analyses. There are two reasons why not all 8648 ART treatments are included. Firstly, the study was carried out before the enrolment of cohort B was finished, and only includes treatments that had been performed between 2007-2017. Secondly, some couples and cycles were excluded due to not fulfilling the specific inclusion criteria of study II (details in paper II).

Studies III-IV only includes ART treatments leading to childbirth. Of the 1964 children in cohort B, 1660 are included in study III and 1772 in study IV. This is because when data were retrieved from NBHW, the MBR was only updated until 31st December 2017, and the NRCA and the NPR until 31st December 2018. This means that ART treatments leading to childbirth after 2017 are not included in study II, and there is no full coverage rate for the children born in 2018 in study IV.

Methods

Randomized placebo-controlled trial

In study I, participants came to a screening visit where semen and blood samples were obtained, weight and height were measured, and health-related questions were asked. Subjects that met the inclusion criteria were randomized to either antioxidant treatment, with a dietary supplement (Androferti©, Q Pharma Laboratorios S.L., Alicante, Spain) which is marketed to improve sperm parameters, or to placebo. Antioxidants and placebo were packed in identical boxes and numbered according to a randomization list provided by the pharmaceutical company that supplied the products. Subjects, researchers, and data collectors were blinded to treatment allocation. Antioxidants or placebo were administered orally twice a day for six months. Semen analyses were performed after three and six months of treatment. At each of these visits, the participants met one of the trial personnel, and possible side effects were noted.

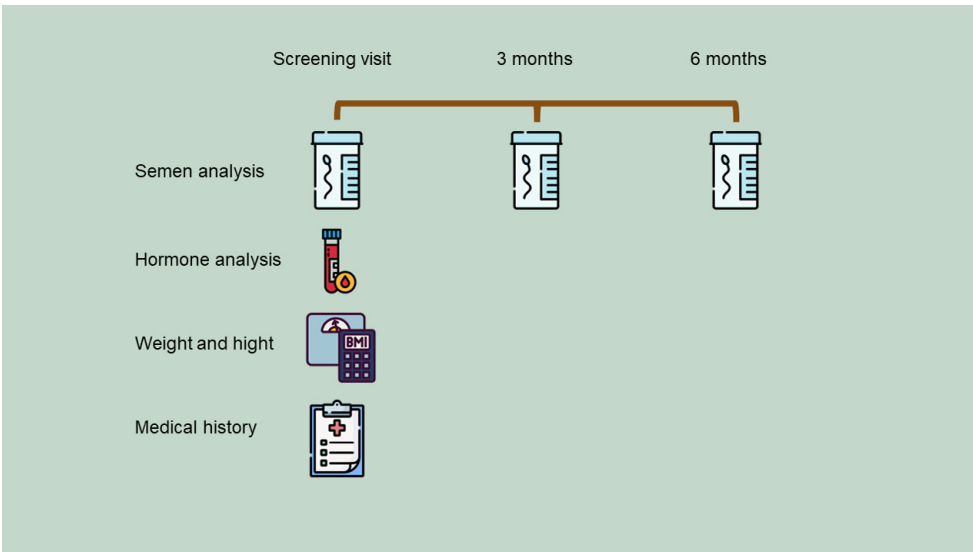

Figure 8. Timeline and data collection in study I.

Blood analysis

For study I, fasting blood samples were obtained from all men at the screening visit. Follicle-stimulating hormone and luteinizing hormone were analyzed by an immunometric sandwich method. Testosterone was measured by a competitive

immunoassay. All samples were obtained fasting, between 8 and 10 a.m., and analyzed at the laboratory of clinical chemistry, Skåne University Hospital, Malmö.

Semen analysis

Semen samples were collected by masturbation. All men were instructed to have an abstinence time of 2-4 days. In study I, the actual abstinence time was recorded. The semen samples in study II-IV were collected on the day of ovum pick up and are the same used for fertilization in the ART treatment.

Conventional semen parameters

Conventional semen analysis was performed in study I. Semen volume, sperm concentration, total sperm count, total and progressive motility, and morphology were assessed according to the WHO guidelines from 2010 (107). All semen analyses were performed at the same laboratory, which serves as a reference in the European Society of Human Reproduction and Embryology external quality control.

Sperm chromatin structure assay

Sperm chromatin structure assay was performed according to a standardized protocol as described by Evenson (13). In short, raw frozen/thawed semen samples were diluted in a buffer to a concentration of 1-2 x 10^6 sperm cells/mL. The diluted samples were exposed to an acid solution with pH 1.2 for 30 seconds and stained with the fluorescent dye acridine orange. Following this, the samples were placed in a flow cytometer. A dedicated software (SCSAsoft®; SCSA Diagnostics, Brookings, USA) was used to analyze the flow cytometric data. For the instrument setting, aliquots from a donor semen sample with normal DFI were used as a reference. This reference sample was run each time before samples were to be analyzed, to calibrate the equipment.

The use of a flow cytometer and computer-based assessment results in good repeatability of the SCSA test and very limited intra-laboratory variability (108). The test also has low inter-laboratory variability. A study compared DFI measurements from the same samples performed in two countries, with a high level of correlation (r = 0.90) (109).

Register data

The National Board of Health and Welfare (NBHW) provides several national medical registries. Data from these registries can be used in research after ethical permission and permission from NBHW. Individuals are identified in the registries by their unique personal identification numbers. This number can be used as a key

variable when matching different registries. The personal identification number is removed from the dataset by the NBHW and replaced by a serial number before sending the register data to the researcher. Hence, the study population is anonymous for the researcher while analyzing the data.

The data in studies III-IV are partly based on national medical registries. In the following section, the registers that have been used are introduced.

The Medical Birth Register

The Medical birth register (MBR) was established in 1973. It provides information on prenatal, delivery, and neonatal care. It is mandatory for all healthcare providers to report data from medical records to the MBR, and the register has coverage for almost 100% of all births in Sweden (110).

The register includes diagnosis codes of the mother and the child according to the International Classification of Diseases (ICD). The diagnosis codes of the mother are provided by the prenatal and delivery care units. All parents are offered a physical examination of their child within the first days after delivery. Diagnosis codes from this examination and the neonatal care unit are reported to the MBR. The register also provides information such as the infant's birth weight, Apgar score, gestational age, sex, and if it was a single or multiple birth. Stillborn children with a gestational length of at least 22+0 weeks (or 28+0 weeks if the child was born before 1st July 2008) are also included in the register.

To estimate the degree of coverage, the reported births in MBR are regularly matched on personal identification numbers with Statistics Sweden's total population register. This shows that missing births in MBR have been below one percent since 2015 (110). Incoming data are quality-checked to find unreasonable, invalid, or contradictory data. Birth weight is considered such a central variable that missing or suspected wrong values are routinely requested from the healthcare units. Still, other values can be requested if missing. Although the register may contain inaccuracies, for example when wrong information is entered into the medical record, it is considered to maintain good quality.

The National Register of Congenital Anomalies

The National Register of Congenital Anomalies (NRCA) contains information about congenital malformations and chromosomal abnormalities for both liveborn and stillborn children born from gestational week 22+0 and induced abortions. It is compulsory to report to the register, and the information is obtained from medical records. Only severe congenital malformations are reported to this register. Since 2013 the data are not only based on cases that are reported directly to the register. The MBR and NPR are also regularly checked to find children with severe malformations that have not been reported to NRCA (111).

The National Patient Register

The National Patient Register contains data on both in-patient and out-patient specialist care. Since 1984, it has been mandatory for all county councils to report to the register. Data on out-patient specialist care have been included since 2001. Both private and public caregivers must report to the register. The register contains information about ICD codes that are obtained from medical records. The reported data are quality checked by the NBHW, and missing or invalid data can be requested from the caregivers (112).

Statistical methods

Study I

Before inclusion in the study, a power calculation was performed. With the assumption of a mean pre-treatment DFI of 32% and a standard deviation of 11%, the power calculation showed that 39 trial participants were required in each arm to detect with 80% power ($\alpha = 5\%$) a 7% difference in change of DFI between the antioxidant and placebo group.

When comparing the antioxidant and placebo groups, Mann-Whitney U-test was applied. This test compares differences between two independent groups and is preferably used when the residuals of the dependent variable are not normally distributed, as often is the case when semen parameters are analyzed. In such cases, non-parametric tests are preferred. The Wilcoxon test for paired data analyzed within-subject changes in sperm characteristics during the treatment period. This test can compare related samples, like repeated measurements from the same subject.

Study II

All couples were divided into two groups according to the first ART-treatment method (referred to as the IVF group and the ICSI group). Secondly, the couples were divided into DFI groups. Sperm DFI ≥ 20% was defined as high since previous research found declined fertility above this threshold (68, 69). Couples that had performed more than one ART treatment were grouped according to the first available DFI value.

Logistic regression was used to analyze the association between DFI and cumulative live birth rate (CLBR). The analyses were adjusted for maternal age.

The aim was to investigate the effect of DFI on CLBR after up to three complete cycles (ovarian stimulation and the resulting fresh as well as all frozen ET). All couples were followed until either a live birth, or until they had used all three complete cycles, or discontinuation for other reasons (lost to follow-up). Both

conservative and optimal CLBR were calculated. The conservative estimate assumes that lost to follow-up couples would not have achieved live birth if they had continued. The optimal estimate assumes that lost to follow-up couples would have had the same LBR as those who continue treatments until live birth or three complete cycles.

Study III-IV

A power calculation was performed before the inclusion of cohort B, used in study II-IV. With the estimation that 25% of all men would have DFI ≥ 20%, 1950 delivery cycles were required to detect an increased risk of an adverse outcome (such as CM) from 3% to 6% with a power of 80% ($\alpha = 5\%$).

Logistic regression was used to examine the association between DFI groups and the different outcomes (PE, PTB, LBW, SGA, LAS, CM, and multiple CM). In the analysis of PE, PTB, LBW, SGA, and LAS, adjustment for paternal age was made.

As in study II, DFI ≥ 20% was used as a cut-off to distinguish between the high and normal DFI groups. Patterns in the proportion of PE across different levels of DFI indicated that other cut-offs provide additional information about the relationship between DFI and PE. Thus, OR for PE based on five 10-percentile groups of DFI, and a comparison of DFI < 10% vs. DFI ≥ 10% were also calculated.

For all outcomes, sensitivity analyses were performed where all multiple birth children and their parents were excluded from the analysis.

Ethics

The studies were approved by the Regional Ethical Review Board in Lund, Sweden (Study I: Dnr. 2014/89; Study II: Dnr. 2015/006; Study III-IV: Dnr. 2015/006 and 2018/24). All analyses were made on anonymized data to protect participant privacy.

Methodological considerations

Study I

Study I is a double-blind, placebo-controlled RCT. This method is considered to be the gold standard in treatment evaluation. By randomization, problems with selection bias and confounding factors can be minimized. Detection bias can occur when the investigator assesses outcome measures differently, depending on the trial participant's treatment group. This kind of bias was reduced by blinding.

Study design and study size were planned with the aim of avoiding type 1 and type 2 errors. Type 1 errors occur when the null hypothesis is rejected, although it is true. Type 2 errors occur when we hold on to the null hypothesis, even though it is not true. To reduce the risk of type 1 error, a significance level of 5% is typically used, and this is also the level used in study I. This means that the probability of type 1 error is a maximum of 5%. To minimize the risk of type 2 error, a power calculation was performed before study inclusion in order to have a sufficient sample size.

Although the RCT methodology has advantages in reducing bias and confounding factors, it also has some disadvantages. It is expensive, time-consuming, and can be a logistic challenge. Dropouts are common in RCTs and are a potential source of bias. Our study had a low dropout rate, and the reason for the discontinuation was requested to better determine whether the dropout could lead to bias.

The influence of funding sources on reporting the study results, referred to as sponsorship bias, is a known risk in all kinds of studies. A Cochrane review showed that industry-sponsored studies had more favorable results and conclusions than studies sponsored by other sources (113). Study I was funded by the pharmaceutical company that sells the tested antioxidant. To minimize sponsorship bias and report bias, a signed agreement was made with the sponsor before the study began, which guaranteed that the pharmaceutical company would not be a part of the data collection or analysis and that they could not object to the publication of the trial result.

Studies II-IV

Studies II-IV are observational cohort studies. These are all based on data from medical records, and studies III-IV include data from national registers as well. This methodology is non-invasive and cost-effective, and large studies can be made.

Selection bias was reduced by having a well-functioning recruitment procedure in the prospective inclusion of the couples to maximize participants' response rates. An "opt-out" model was used in the retrospective inclusion. In summary, this led to a high rate (94%) of included couples.

Loss to follow-up can be a source of bias if the likelihood of loss to follow-up is related to exposure and outcome in the study. In study II, this was addressed in the choice of statistical method, where both conservative and optimal estimates were calculated.

Another type of bias to consider is information bias. An example is if a particular group is more likely to have missing data. Since the data in study II are based on medical records, no data were missing regarding pregnancy outcomes. Studies III-IV include data from national registries. As described in a previous section, the MBR has almost 100% coverage of births in Sweden (110). Still, the information in the register can be incomplete, with missing data and diagnoses. It is unknown how

extensive the data loss is regarding PE and CM diagnosis in MBR. However, if the lack of data is random, it has little effect on risk estimates. Data regarding CM diagnoses were also obtained from two additional registries, NRCA and NPR, to identify cases with missing data in MBR.

In all observational studies, confounding can result in inaccuracy in the measure of association between exposure and outcomes. To be a confounder, the variable must be associated with both the outcome and the exposure and be unequally distributed between exposure groups. Additionally, it should not be a part of a casual pathway (as a mediator). In study II, analyses are adjusted for female age. Female age is associated with the outcomes of the study (miscarriage, fertilization rate, and CLBR). Previous research has shown that the impact of high DFI (the exposure in study II) on IVF outcomes is more pronounced if the woman has low AMH levels (114). Advanced female age is associated with lower AMH levels (115). This means that female age indirectly could be associated with the effect of high DFI. Analyses in study III are adjusted for paternal age. The outcomes of the study, preeclampsia and adverse perinatal outcomes, are associated with paternal age (96, 116). Advanced age is a risk factor for high DFI (51). No adjustment for paternal age was performed in study IV since there is no association between CM (the outcome) and paternal age < 55 years (unpublished data, based on all children in MBR born between 1994-2014).

To minimize the risk of type 2 error, a power calculation was performed before the enrollment of participants.

Results

Study I

Comparing pre-treatment values with DFI levels at three and six months of treatment within the antioxidant group, no statistically significant difference in DFI was seen during the trial period (Fig 9).

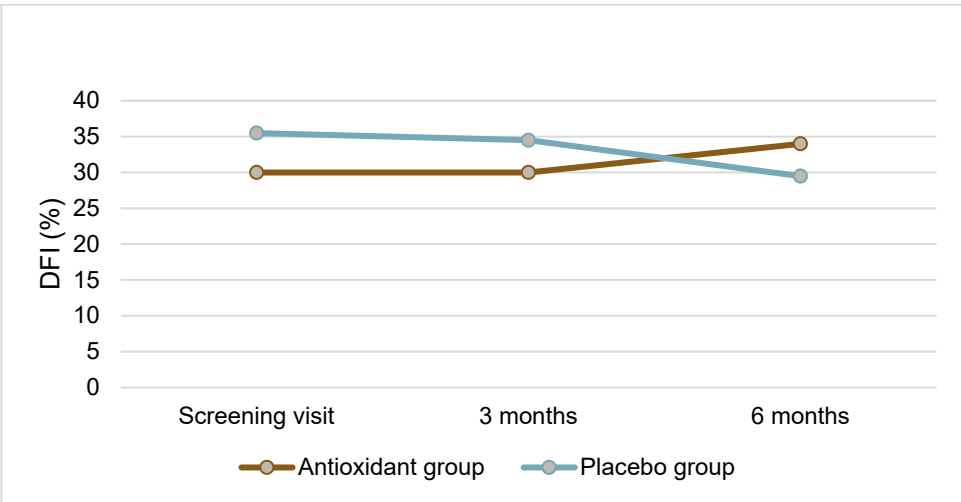

Figure 9. DNA fragmentation index at baseline, three months, and six months of antioxidant treatment or placebo.

Note: DFI = DNA fragmentation index.

DFI is presented as medians.

No statistically significant differences between the antioxidant and the placebo group were found for any of the semen parameters at any of the three visits (pre-treatment, at three months, or six months).

Analyzing within-group changes, as compared to pre-treatment values, the antioxidant group had higher sperm concentration after three months of treatment (median: 24.4 × 10^6/mL vs. 27.2 × 10^6/mL; p = 0.028) and borderline statistically significant higher sperm concentration after six months of treatment (median: 24.4 × 10^6/mL vs. 33.3 × 10^6/mL; p = 0.053). Semen volume was decreased in the

antioxidant group after six months of treatment (median: 3.84 mL vs. 3.35 mL; p = 0.026). No statistically significant changes were seen in the antioxidant group as considers total sperm count or motility.

Study II

In the IVF group, couples with DFI < 20% had higher CLBR compared to DFI ≥ 20% (Fig 10). There was a relative difference in CLBR of 16% for the conservative estimate and 8% for the optimal estimate. The difference was statistically significant for both estimates in an unadjusted model (p = 0.042; p = 0.019 for the conservative and the optimal estimates, respectively) and in the optimal estimate after adjustment for female age (p = 0.115; p = 0.045 for the conservative and the optimal estimates, respectively). No DFI-dependent difference in CLBR was seen in the ICSI group.

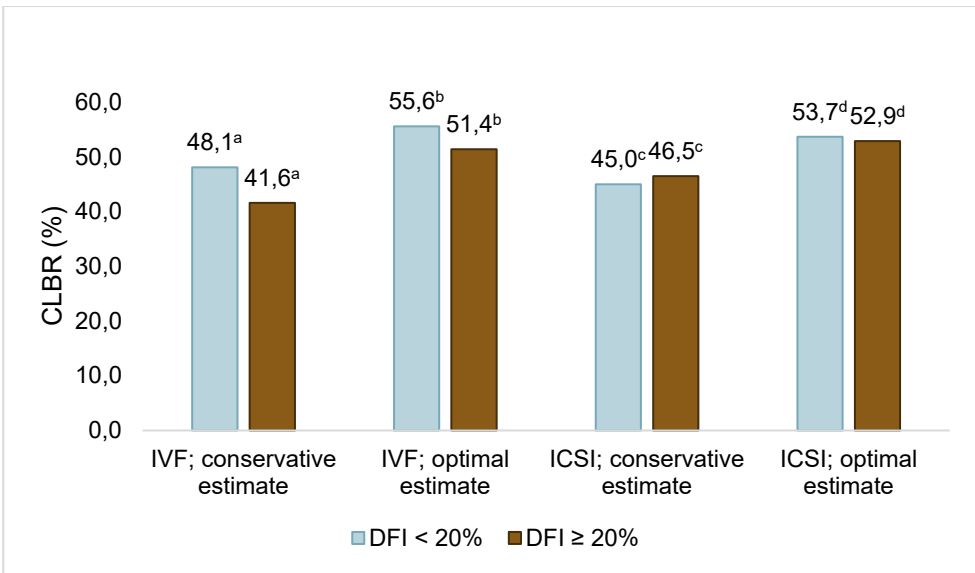

Figure 10. Cumulative live birth rate according to DFI (< 20% vs. ≥ 20%) and method of fertilization in the first treatment cycle (IVF vs. ICSI). Both conservative and optimal estimates are presented.

Note: CLBR = cumulative live birth rate; DFI = DNA fragmentation index; ICSI = intracytoplasmic sperm injection; IVF = in vitro fertilization.

a) Unadjusted/adjusted comparison of DFI < 20% vs. DFI ≥ 20% for the conservative CLBR, p = 0.042/0.115.

b) Unadjusted/adjusted comparison of DFI < 20% vs. DFI ≥ 20% for the optimal CLBR, p = 0.019/0.045.

c) No statistical significance in either the unadjusted or adjusted comparison of DFI < 20% vs. DFI ≥ 20% for the conservative CLBR.

d) No statistical significance in either the unadjusted or adjusted comparison of DFI< 20% vs. DFI ≥ 20% for the optimal CLBR.

Study III

The association between DFI and PE

With 20% DFI cut-off for the entire cohort, there was no statistically significant difference in the risk of PE. However, when analyzing IVF and ICSI treatments separately, the OR of PE was 2.2 (95% CI 1.1 - 4.4) for the IVF-treated women, with 10.5% of them being diagnosed with PE if DFI ≥ 20% compared to 4.8% in the DFI < 20% group (Fig 11). DFI was not associated with PE in the ICSI group.

When the couples were divided into five groups according to DFI value, with DFI < 10% as a reference, the PE risk increased in IVF pregnancies in a dose-response manner already at DFI levels ≥ 10% (Table 2).

Comparing DFI < 10% and DFI ≥ 10%, OR of PE was statistically significantly increased in the high DFI group, both in the total cohort (OR = 2.1; 95% CI 1.2 - 3.8) and in the IVF group (OR = 2.3; 95% CI 1.1 - 4.8), but not in the ICSI group (OR = 1.8; 95% CI 0.61 - 5.1).

The statistically significant increases in the OR for PE, both in the total cohort and in the IVF group, were robust to exclusion of all multiple births when 10%, but not if 20%, was used as DFI cut-off.

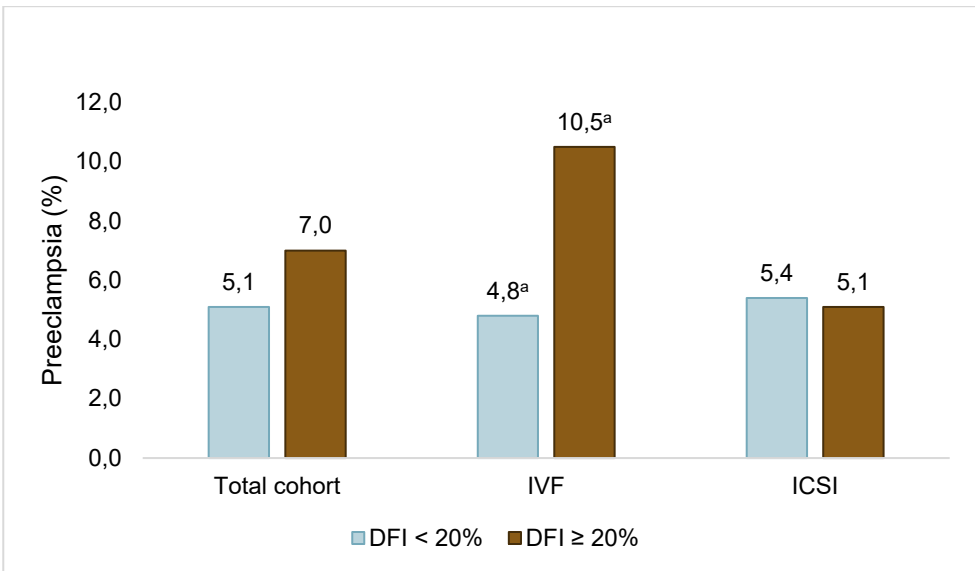

Figure 11. Risk of preeclampsia by DFI (< 20% vs. ≥ 20%) and method of fertilization.
Note: DFI = DNA fragmentation index. ICSI = intracytoplasmic sperm injection. IVF = in vitro fertilization. The unit of observation is the couple.

a) p = 0.02, adjusted for paternal age.

Table 2. Risk of preeclampsia by DFI groups and method of fertilization.

	DFI <10%	DFI ≥10% & <20%	DFI ≥20% & <30%	DFI ≥30% & <40%	DFI ≥40%
Total (N=1594)					
Preeclampsia	3.1% (14/450)	6.2% (48/772)	6.2% (15/242)	8.0% (7/87)	9.3% (4/43)
Adj. OR (95 % CI)	Ref.	2.0 (1.1 - 3.7)	2.0 (0.95 - 4.2)	2.6 (1.0 - 6.8)	3.0 (0.92 - 9.6)
IVF (N=841)					
Preeclampsia	3.1% (10/325)	6.2% (25/402)	8.7% (7/80)	10.7% (3/28)	33.3% (2/6)
Adj. OR (95 % CI)	Ref.	2.0 (0.95 - 4.3)	2.9 (1.1 - 7.9)	3.5 (0.89 - 14)	13.7 (2.2 - 86)
ICSI (N=741)					
Preeclampsia	3.3% (4/122)	6.1% (22/362)	4.9% (8/162)	5.2% (3/58)	5.4% (2/37)
Adj. OR (95 % CI)	Ref.	1.9 (0.64 - 5.7)	1.5 (0.45 - 5.2)	1.6 (0.35 - 7.4)	1.7 (0.29 - 9.7)

Note: DFI = DNA fragmentation index. ICSI = intracytoplasmic sperm injection. IVF = in vitro fertilization. OR = odds ratio. CI = confidence interval. Ref. = reference category. The odds ratios are adjusted for paternal age. The unit of observation is the couple.

The association between DFI and adverse perinatal outcomes

In the entire cohort, DFI ≥ 20% was associated with an increased OR of PTB (OR 1.4; 95% CI 1.0 - 2.0; p = 0.03). Without reaching the level of statistical significance, similar risk estimates were seen in both the IVF (OR 1.5; 95% CI 0.84-2.5) and the ICSI group (OR 1.5; 95% CI 0.94-2.3). The statistically significant increased OR of PTB in the high DFI group in the total cohort remained after the exclusion of all multiple births. Preterm birth rates for the two DFI groups are presented in Figure 12.

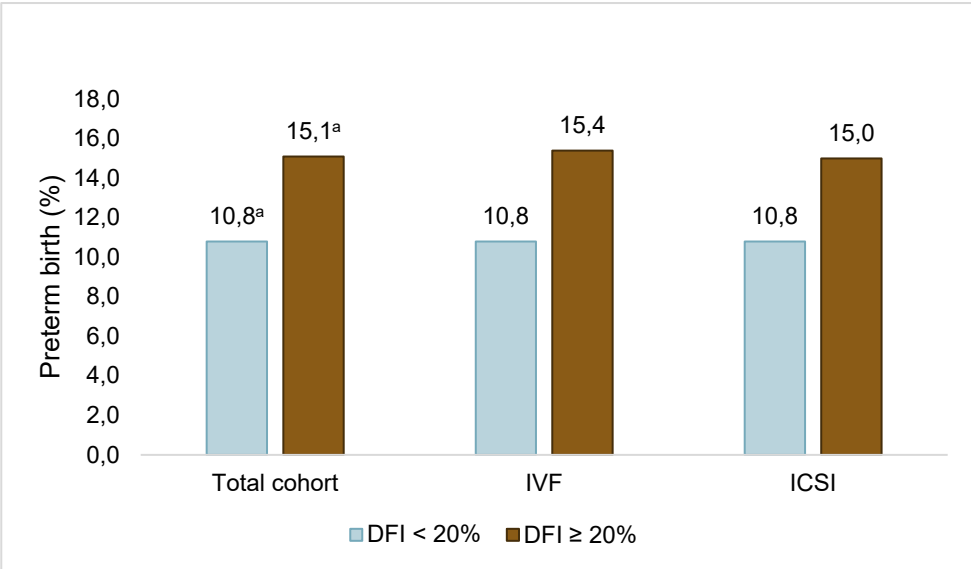

Figure 12. Preterm birth by DFI (< 20% vs. ≥ 20%) and method of fertilization.
Note: DFI = DNA fragmentation index. ICSI = intracytoplasmic sperm injection. IVF = in vitro fertilization. The unit of observation is the child.

a) p = 0.03, adjusted for paternal age.

There were no significant differences in ORs for LBW, SGA, or LAS between the two DFI groups.

Study IV

The high DFI group had an increased risk of having both major malformations (OR 1.5; 95% CI 1.0 - 2.3; p = 0.046) and multiple malformations (OR 2.1; 95% CI 1.2 - 3.7; p = 0.010). The findings were robust to the exclusion of all multiple birth children. When analyzing IVF and ICSI separately, the effect of DFI on major and

multiple malformations was approximately the same regardless of treatment type and not statistically significant (Fig. 13 & 14).

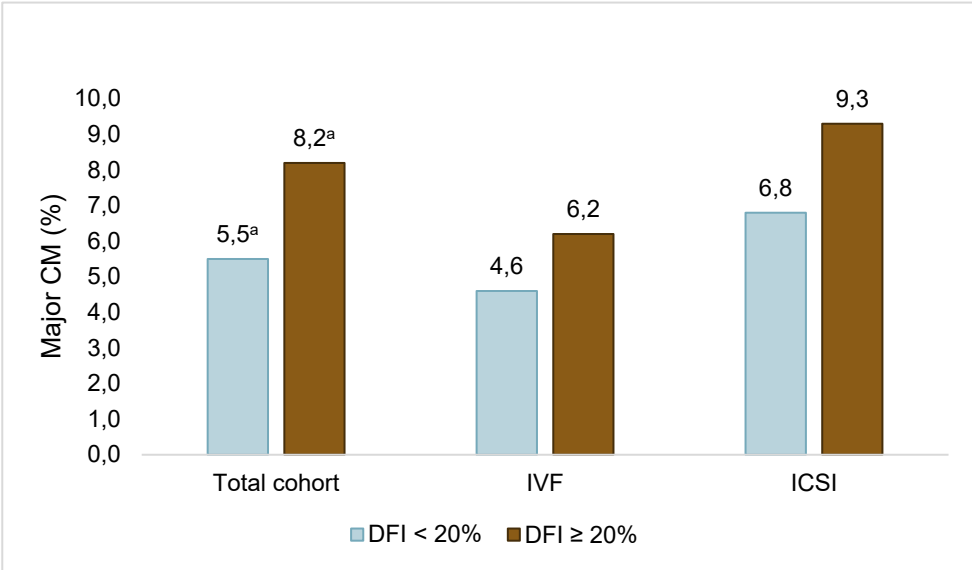

Figure 13. Major congenital malformations by DFI (< 20% vs. ≥ 20%).
Note: DFI = DNA fragmentation index. CM = Congenital malformation.
a) p = 0.046

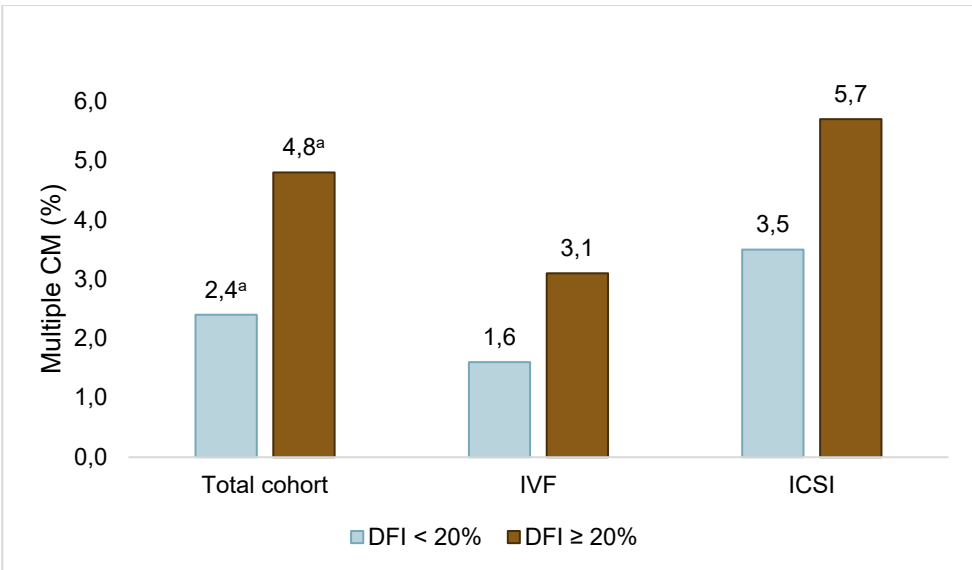

Figure 14. Multiple congenital malformations by DFI (< 20% vs. ≥ 20%).
Note: DFI = DNA fragmentation index. CM = Congenital malformation.
a) p = 0.010

Discussion

The main finding of this thesis is that high DFI has a negative impact not only on the success rate of infertility treatments but also on pregnancy outcomes and the health of the offspring. Our findings provide new knowledge that is of clinical importance and give us a better understanding of the underlying biological mechanisms behind the adverse outcomes studied.

Since the first IVF baby was born in 1978, significant progress has been made in making ART treatments effective. Despite this, approximately one in three couples remain childless after the three IVF or ICSI cycles offered to a couple as a part of the public healthcare in Sweden. Further, women pregnant by ART have an increased risk of preeclampsia, and the children are at higher risk of preterm birth, intrauterine growth restriction, and congenital malformations (82, 99). Infertility and the use of ART are increasing. Consequently, it is essential to continue the work to make ART treatments as effective and safe as possible and to explore the underlying mechanisms behind the increased risk of adverse pregnancy and perinatal outcomes following ART.

This thesis aims to investigate the clinical impact of DFI on cumulative live birth rate, adverse pregnancy and perinatal outcomes, and the offspring's health.

We found that high sperm DFI was associated with lower CLBR and higher rates of PE in IVF cycles. No DFI-dependent differences in CLBR or PE were seen in the ICSI group. When analyzing the whole cohort, both IVF and ICSI treatments included, there was an increased risk of PTB and CM in the high DFI group. Further, we found that antioxidant treatment did not affect DFI in infertile men with high DFI.

The finding of study II, that high DFI was associated with lower LBR in ICSI cycles compared to standard IVF, is in agreement with previous findings (117-119). However, these studies have focused on the effect of DFI on single fresh cycles, and not on the CLBR. From a clinical point of view, CLBR is a preferable measure of the success of ART treatments. The novelty of our study is that it mirrors a real-life setting in fertility clinics, including up to three complete cycles for every couple. Most couples undergo more than one treatment and often a mixture of fresh and frozen ETs. Some couples switch between treatment types; in case of poor fertilization or treatment failure, IVF can be altered to ICSI. Thus, an important question is whether introducing DFI as a new criterion in the decision-making

regarding the first treatment type will have any impact on CLBR. In order to increase CLBR, our findings suggest that ICSI may be preferable instead of IVF in the case of DFI ≥ 20%.

It has been described previously that ART-pregnant women are at higher risk of PE than naturally conceived (83). Oocyte donation and FET in a programmed cycle are known ART-related risk factors of PE (120, 121). It has been hypothesized that the absence of corpus luteum is a part of the underlying mechanism (122). However, not only maternal but also paternal factors play a role in the onset of PE (90), but it is not known by which biological mechanism. Advanced paternal age has been associated with a higher risk of PE (116), and it has been speculated whether this acts through sperm DNA damage (91). Our study is the first to show such an association. The relationship between DFI and PE in IVF cycles showed a dose-response-like effect and started at DFI levels exceeding 10%.

The placenta is genetically derived from both the father and the mother and is the cornerstone of the pathophysiology of PE. Preeclampsia is associated with a lower placenta weight. The link between DFI and PE is supported by an animal study showing lower placenta weight with high SDF (123). Further, a study by Hoek *et al.* reported higher placenta weight with the use of testicular sperm (124), which often have lower SDF than ejaculated sperm (60).

No DFI-dependent differences were seen in CLBR or PE in the ICSI groups. In papers II and III, three possible explanations of this finding were suggested. In ICSI, a spermatozoon with normal morphology is chosen by the embryologists for fertilization (125). This active selection might result in a less DNA-damaged spermatozoon. Secondly, ICSI is preferably done in case of male infertility. It can be assumed that the women in this group have a better fertility status and oocytes with a better DNA repair capacity. The third point is that different culture environments are applied for IVF and ICSI. In IVF, the oocyte is co-incubated with spermatozoa and can be exposed to ROS, catabolites, and microbes during incubation. In ICSI, the spermatozoon is injected directly into the oocyte, and the gametes are probably less exposed to ROS.

It is well-known that the risk of CM is increased in ART-conceived children compared to naturally conceived (99). The results of study IV show an increased risk of CM in children conceived with ejaculates with high DFI, suggesting that high DFI could be one of the underlying mechanisms behind the association between the use of ART and CM. Paternal smoking (104) has been linked to an increased risk of CM in children, and it has been speculated that sperm DNA damage is one of the mechanisms behind this finding. Our study is the first to show a direct association between DFI and the risk of CM.

Previous studies show that ART-conceived children have more de novo mutations, most of which originated from the father (126). De novo mutations are a well-known cause of congenital diseases and malformations. Further, spermatozoa with

chromosomally unbalanced malformations are more likely to have fragmented DNA (127). There are very limited previous studies about the health effects of SDF in the offspring. In 2020, a study of 713 ICSI delivery cycles was published (81), and the authors stated that there was no difference in CM risk between DFI groups (DFI<15%, 15%-30%, and >30%). However, in the cohort of 984 children, there were only seven cases of CM. That means only 0,7% of the children were reported to have a CM, significantly fewer than the 6% estimated by WHO (97). An underreporting can be suspected, and it is difficult to draw any conclusions with the small number of children with CM in that study. Further, DFI was not analyzed in the sperm sample used for fertilization but in another sample collected pre-treatment.

Our study shows a higher CM rate than the prevalence reported by EUROCAT. Partly, this can be explained by the known increased CM risk in ART-conceived children. Another explanation is that we have included CM diagnoses not only from the MBR but also from the NRCA and the NPR. By doing this, better coverage was obtained. The child of the children in the MBR come from the neonatal care unit and the newborn physical evaluation, typically performed within 24 hours of age. Congenital malformations are not always obvious at this first physical evaluation and might show later (e.g. some cardiovascular malformations) or be referred for a second opinion (e.g. hip dysplasia). In such a case, no diagnosis is made in MBR. Instead, the child might be diagnosed later, and the CM diagnosis is then found in the NPR. With our methodology, we believe that the data are more complete, but the CM prevalence cannot be compared with that based on studies that obtained data from MBR alone.

An obvious strength of studies II-IV is the large cohort on which the results are based. As discussed in the section “Methodological considerations”, a high rate (94%) of the eligible couples were included in the cohort. Further, the national medical registries used are considered to maintain high quality. Studies II-IV are all observational cohort studies. Although this methodology is well suited for the research questions and has made it possible to examine relatively rare outcomes, a challenge of observational cohort studies is to rule out confounding factors. When planning the statistical analyses, directed acyclic graphs were used to identify confounding variables, but unknown confounders cannot be excluded.

As with conventional sperm parameters, there is an intra-individual variability in DFI. In many studies, DFI is measured prior to ART treatment. To provide better accuracy, all DFI-values in cohort B were analyzed in the actual sperm sample used for the ART treatment.

In studies II-IV, high DFI is associated with a lower success rate in IVF cycles and adverse pregnancy and perinatal outcomes. These results highlight the importance of further investigating the possibilities of treating high DFI. Study I evaluate the effect of combined antioxidant treatment on subfertile men with high DFI. Six

months of treatment did not significantly improve DFI or the conventional sperm parameters. At the time the study was performed, to our knowledge, only one randomized placebo-controlled trial investigating the effect of antioxidants on DFI had been published, showing a decrease of DFI with vitamin C and E treatment (53). Since then, a few more antioxidant RCTs have been performed, some showing a reduction in SDF (56, 57) and some with no effect on SDF (54, 55). The diversity in results could have many reasons. All studies have used different antioxidants, dosages, and treatment lengths and have used different inclusion criteria for the trial participants. We excluded all smokers and obese men. These conditions are associated with increased oxidative stress (128), meaning we might have excluded men who would benefit from antioxidant treatment.

Study I had DFI as a primary outcome. From an infertile couple's point of view, however, DFI is not the most important. The ultimate end-point for an infertile couple is a healthy pregnancy leading to a live birth. A limitation of our study is that we did not have pregnancy or live birth rates as outcomes, which instead could be the focus of a future study.

Clinical implications

Our results suggest that including DFI in the decision-making regarding fertilization method and performing ICSI instead of IVF in the case of DFI ≥ 20% could improve CLBR and decrease the risk of PE for this group of patients. This could result in fewer treatments, reduce medical risks and economic costs, and improve the overall results of fertility clinics. In recent years, there has been a trend towards increased use of ICSI, also in non-male factor infertility. In Sweden, this trend has been less pronounced than in many other countries, and 50 % of all treatments are standard IVF (105). A possible overuse of ICSI is not unproblematic. There are concerns since ICSI bypasses the natural selection of spermatozoa. Further, the technique is time-consuming and more expensive. Therefore, it is essential to locate the subgroups that might benefit from this more invasive method of fertilization. Our summarized findings suggest that couples with high DFI are one of these subgroups.

Further, we found an increased risk of CM in the high DFI group. Finding risk factors of CMs enables a better risk assessment in early pregnancy. In the case of several factors associated with CMs, it might be relevant to perform a targeted ultrasound during pregnancy for the detection of fetal structural abnormalities.

Conclusions

The summarized finding of this project suggests that including DFI in infertility evaluation and in decision-making regarding choice of ART treatment is beneficial for the patients in their journey towards their ultimate goal – a healthy pregnancy resulting in a live birth.

The detailed conclusions from the studies are:

I. Six months of treatment with combined antioxidants had no effect on DFI in men from infertile couples with normal reproductive hormone levels and DFI ≥ 25%.

II. DFI ≥ 20% predicts lower CLBR if IVF and not ICSI is applied in the first cycle of ART.

III. DFI ≥ 20% was associated with an increased risk of PTB and, in IVF pregnancies, an increased risk of PE. The risk of PE in IVF-pregnant women was increased already at DFI ≥ 10%, and the OR of PE increased with increasing DFI in a dose-response manner.

IV. In children conceived by ART, DFI ≥ 20% was associated with an increased risk of CM.

Future perspectives

In study II, CLBR is lower in the high DFI group when IVF is performed in the first treatment cycle. To verify the results, the ideal would be a prospective study where couples who are fulfilling the criteria for standard IVF but have high DFI, are randomized for either IVF or ICSI.

The study population of study III-IV consists of infertile couples and their ART-conceived children. This means that the result might not be generalizable to naturally conceived. An increased risk of PE was seen already at DFI > 10%, a level compatible with natural fertilization. It would be interesting to see if there is an association between DFI and PE in those who are spontaneously pregnant as well.

Women undergoing treatment with donated oocytes or FET in a programmed cycle have an increased risk of PE. Investigating the association between DFI and PE in these high-risk subgroups is important. Is there an additive risk? Or does one plus one equal three in terms of PE risk?

Our findings highlight the importance of developing treatment strategies to reduce DFI. In study I we did not see any effect of antioxidant treatment on DFI. Further RCTs are needed to clarify the effect of antioxidants on DFI and live birth rates in different subgroups of subfertile men.

The increased risk of adverse outcomes in the high DFI group leads us to the question of whether treatment of DFI would result in reduced risk of PE, PTB, and CM. Future studies are needed to evaluate this.

ART-conceived children are at higher risk of CM and adverse perinatal outcomes such as PTB and LBW. Studies on long-term health are limited. The first IVF child was born in 1978, and the first ICSI child in 1992, which means that most ART-conceived still are children or in their early adulthood. Surveillance of the long-term health of ART-conceived is essential.

Sammanfattning på svenska

Ungefär ett av sju olikkönade par har problem med att uppnå graviditet. Efter ett års försök utan att befruktning har skett uppfylls kriteriet för infertilitet. Orsaken till barnlösheten ligger lika ofta i manliga faktorer som i kvinnliga, men ibland hittas inga orsaker trots barnlöshetsutredning.

I samband med en barnlöshetsutredning får mannen lämna ett spermaprov. I den konventionella spermaanalysen undersöks spermiekoncentration samt spermiernas rörlighet och form. Detta test har dock en bristande träffsäkerhet. Det finns män som har ett normalt spermaprov enligt analysen, men ändå har nedsatt fertilitet. Spermiens uppgift är att transportera mannens arvsmassa (DNA) till kvinnans ägg, så att befruktning kan ske. DNA kan ha olika kvalitet, och det har visat sig att det är vanligt med DNA-skador i spermierna hos infertila män. Om andelen spermier med DNA-skador överstiger 30%, är chansen till naturlig befruktning nästintill noll. Detta har gjort att det har utvecklats metoder för att mäta andelen spermier med DNA-skador, med ett mått som kallas för DNA fragmenteringsindex (DFI). Sådan analys görs på vissa fertilitetskliniker som ett komplement till den konventionella spermaanalysen, men på de flesta kliniker är DFI inget som analyseras på rutin.

Lyckligtvis har det de senaste decennierna gjorts stora framsteg beträffande olika fertilitetsbehandlingar, något som medför att många infertila par kan få sitt efterlängtade barn. Det finns två olika typer av provrörsbefruktningar: IVF och ICSI. Vid IVF läggs ägget i en liten skål tillsammans med ett större antal spermier som själva får ta sig till ägget och befrukta detta. Vid ICSI väljs en enskild spermie ut och injiceras in i ägget. ICSI väljs företrädelsevis när det finns få spermier eller spermierna inte rör sig normalt.

Det finns studier som har visat att ICSI kan ge bättre resultat än med IVF för de par där mannen har förhöjd andel DNA-skadade spermier. De flesta studier som undersökt detta är dock små och baserar sig bara på resultatet av en enstaka fertilitetsbehandling. Infertila som uppfyller vissa kriterier är i Sverige berättigade till tre landstingsfinansierade IVF- eller ICSI-cykler. Målet med delarbete II i denna avhandling var att undersöka hur DNA-skador i spermier påverkade den kumulativa chansen till barnafödelse, efter tre kompletta provrörsbefruktningar. Bland de par som hade gjort IVF som första behandling, hade de med högt DFI (en hög andel DNA-skadade spermier) lägre kumulativ chans till barnafödelse än de med normalt

DFI. Bland paren som gjort ICSI som första behandling var det däremot ingen skillnad mellan de båda DFI-grupperna.

Ända seden det blev klarlagt att en spermie med DNA-skador faktiskt kan befrukta ett ägg i samband med provrörsbefruktning så har det funnits en oro för hur det påverkar de barnen som blir till. Det är känt sedan tidigare att barn som blivit till med hjälp av provrörsbefruktning har en något ökad risk för missbildningar, att födas för tidigt och vara små för tiden vid födseln. Den bakomliggande orsaken är inte klarlagd. Känt är även att kvinnor som blir gravida efter fertilitetsbehandling löper en ökad risk för havandeskapsförgiftning.

I delarbete III undersöks sambandet mellan DFI och havandeskapsförgiftning, för tidig födsel och andra negativa utfall i samband med födelse (såsom att vara liten för tiden). Delarbete IV undersöker sambandet mellan DFI och risken för missbildningar hos barnen. Förhöjd andel DNA-skadade spermier är kopplat till ökad risk för havandeskapsförgiftning, för tidig födsel och missbildningar. Den förhöjda risken för havandeskapsförgiftningar sågs bara i gruppen som gjort IVF, inte bland dem som gjort ICSI. Rörande för tidig födsel och risk för missbildningar var det ingen skillnad mellan behandlingsgrupperna.

Eftersom högt DFI verkar påverka både resultatet av provrörsbefruktning och barnens hälsa, är det naturligt att fråga sig om det går att behandla högt DFI på något sätt. Det finns flera olika sjukdomar samt livsstils- och miljöfaktorer som har visat sig öka risken för DNA-skador i spermier. Detta sker bland annat genom oxidativ stress, ett tillstånd som uppstår vid en obalans mellan antioxidanter och fria syreradikaler. De fria syreradikalerna anses skada spermiernas DNA och på så sätt ge upphov till ett högre DFI. I delstudie I undersöks effekten av antioxidantbehandling på DFI i en grupp av män med nedsatt fertilitet. Runt hälften av männen fick antioxidanter i sex månader, den andra hälften fick placebo. Ingen skillnad i DFI sågs mellan placebo- och antioxidantgruppen under studiens gång.

Sammanfattningsvis pekar de inkluderade studiernas resultat på att förhöjt DFI negativt påverkar chansen att få barn efter provrörsbefruktning och ökar risken för havandeskapsförgiftning, för tidig födsel och missbildningar hos barnen. Det var bara i gruppen som gjorde IVF som första fertilitetsbehandling som det syntes en minskad chans till barnafödsel vid högt DFI. I ICSI-gruppen var det ingen skillnad i chans till barnafödsel mellan de med hög respektive normal DFI. Vidare var det bara i IVF-graviditeter som vi kunde se en ökad risk för havandeskapsförgiftning. Eftersom förhöjd andel DNA-skadade spermier gav sämre resultat och även ökad risk för havandeskapsförgiftning vid IVF och inte vid ICSI, talar våra resultat för att ICSI är att föredra framför IVF då mannen har förhöjt DFI.

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