# *Value of Chromomycin A3 Staining in Assisted Reproductive Technologies*

**Saartje Bloemen**

promotor :
dr. Willem OMBELET
Prof. dr. Lena DE RYCK

co-promotor :
M. NIJS

Universiteit Maastricht

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## List of abbreviations

WHO: World Health Organization

ZP: zona pellucida

DNA: deoxyribonucleic acid

ART: assisted reproductive technologies

IUI: intrauterine insemination

IMC: inseminating motile count

IVF: in vitro fertilization

hCG: human chorionic gonadotropin

OPU: oocyte pick-up

COC: cumulus oocyte complex

ICSI: intracytoplasmic sperm injection

RNA: ribonucleic acid

P1: protamine 1

P2: protamine 2

$CMA_3$: chromomycin $A_3$

TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

HDS: high DNA stainability

rpm: rotations per minute

EBSS: Earle's balanced salt solution

HSA: human serum albumin

PVP: polyvinyl pyrrolidone

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## Samenvatting

De kwaliteit van humaan sperma wordt getest door verschillende conventionele parameters zoals concentratie, morfologie, motiliteit en vitaliteit van de spermatozoa te beoordelen. Deze parameters geven echter niet altijd een indicatie van het bevruchtend vermogen van de spermacellen. Andere factoren, zoals DNA protaminatie hetgeen leidt tot chromatine condensatie, zouden hier ook een rol in kunnen spelen. Tijdens deze studie zal onderzocht worden of DNA protaminatie een bijdrage kan leveren als extra parameter in de evaluatie van het sperma van patiënten. Relaties tussen de DNA protaminatie en de conventionele parameters worden bestudeerd. De relatie tussen DNA protaminatie, spermakwaliteit en de kans op bevruchting en zwangerschap na IUI, IVF of ICSI wordt ook geanalyseerd. De hypothese is dat er een correlatie bestaat tussen minstens één conventionele parameter en DNA protaminatie en dat er een correlatie is tussen DNA protaminatie, spermakwaliteit en de kans op bevruchting en zwangerschap.

Concentratie, motiliteit en vitaliteit van de spermatozoa werden bepaald volgens de richtlijnen van de Wereld Gezondheidsorganisatie. Morfologie werd geëvalueerd volgens de strict criteria. DNA protaminatie werd geanalyseerd door middel van de chromomycine $A_3$ kleuring. Tijdens de evaluatie van de fluorescentie werd ook de morfologie van de koppen van de spermatozoa geëvalueerd. Deze parameters werden geëvalueerd voor patiënten die een eerste IUI, IVF of ICSI behandeling ondergingen.

Na evaluatie van $CMA_3$ fluorescentie en morfologie van de koppen van spermatozoa kan besloten worden dat er een significant verschil is tussen macrocefale (92.7% fluorescent), amorfe (39.8%) en normale spermatozoa (17%). De IVF/ICSI groep werd op twee tijdstippen geëvalueerd, namelijk 5-7 dagen voor eicel aspiratie en de dag van eicel aspiratie. WHO parameters, slechte protaminatie en morfologie van de koppen waren statistisch niet verschillend op de twee tijdstippen. Voor de volledige studiegroep werd een negatieve correlatie gevonden tussen slechte protaminatie en totale motiliteit ($R=-0.34307$, $p=0.03$). Voor de IVF/ICSI groep werden er correlaties gevonden tussen $CMA_3$ en morfologie van de koppen (T1: $R=-0.58202$, $p=0.02$; T2: $R=-0.54163$, $p=0.03$) en op T2 tussen $CMA_3$ en morfologie volgens de strict criteria ($R= -0.48807$, $p=0.04$).

Het voortzetten van deze prospectieve cohort studie zou verder kunnen aantonen dat een selectie van de meest normale spermatozoa nodig is voor IUI, IVF en ICSI behandeling. De correlaties tussen alle parameters op de twee tijdstippen in de IVF/ICSI groep zouden erop kunnen wijzen dat elke parameter specifiek is voor een patiënt en/of eenzelfde spermatogene golf.

## Summary

Human semen quality is tested by assessing parameters such as concentration, morphology, motility and viability of the spermatozoa. However, these parameters do not always correlate with the fertilizing potential of sperm cells. Other factors such as DNA protamination, which induces chromatin condensation, can influence the fertilizing ability. This study will investigate whether DNA protamination could play a role as a parameter in the semen evaluation process of infertile patients. Throughout the study relationships between sperm morphology, viability, concentration and motility and the nuclear maturity of human spermatozoa will be investigated. Also the relationship between chromomycin $A_3$ staining, sperm quality and the effect on fertilization and pregnancy rate of patients after IUI, IVF or ICSI will be looked into. It is hypothesized that there will be a correlation between at least one of the conventional parameters and DNA protamination and that there will be a correlation between DNA protamination, sperm quality and the fertilization and pregnancy rates of the patients.

Sperm concentration, motility and viability are determined according to WHO criteria. Sperm morphology is assessed according to the strict criteria. To investigate DNA protamination chromomycin $A_3$ staining is used. Simultaneously with evaluation of chromomycin $A_3$ fluorescence, sperm head morphology was scored. All these parameters were evaluated in patients at a first IUI, IVF or ICSI cycle.

After evaluating chromomycin $A_3$ fluorescence and sperm head morphology it was found that macrocephalic spermatozoa (92.7%) display fluorescence significantly more than, amorphous sperm cells (39.8%) and normal spermatozoa (17%). The IVF/ICSI group was evaluated on two time points, 5-7 days before oocyte pick-up and the day of oocyte pick-up. WHO parameters, poor protamination and sperm head morphology were all similar at the two time points. In the overall population a negative correlation was found between $CMA_3$ positivity and total motility ($R=-0.34307$, $p=0.03$). Moreover, in the IVF/ICSI group correlations were found between $CMA_3$ and sperm head morphology (T1: $R=-0.58202$, $p=0.02$; T2: $R=-0.54163$, $p=0.03$), on T2 $CMA_3$ correlated negatively with morphology (strict criteria) ($R= -0.48807$, $p= 0.04$).

The continuation of this prospective cohort study can further demonstrate that selection of the normal sperm cells is necessary for treatment with IUI, IVF and ICSI. The correlations between all parameters at the two time points may indicate that these parameters are specific for each patient and/or the same spermatogenic wave.

# 1 Introduction

Infertility is present over the entire world, about 15% of couples who want to have a child face fertility problems. Several causes can lie at the origin of infertility, but 30% of the cases are linked to male infertility [1]. During this study a closer look will be taken at male infertility by examining different parameters of a semen sample. In order to investigate characteristics of a semen sample several tests have been developed and threshold values for these tests have been defined. The most widely used tests to evaluate different sperm parameters are those described by the World Health Organization (WHO) (1999). Some examples of sperm parameters are: motility, morphology, concentration and viability of spermatozoa as well as pH and volume of semen samples. Unfortunately, these parameters do not always investigate the functionality of spermatozoa [2]. However, new techniques have been developed to evaluate different sperm functionalities like the acrosome reaction, the presence or absence of zona pellucida receptors (ZP) on the membrane of spermatozoa, binding of the sperm cell to the oocyte as well as tests on deoxyribonucleic acid (DNA) integrity in the head segment of spermatozoa. The latter includes tests for analysis of DNA fragmentation, DNA packaging and correct protamination of sperm DNA [3-7]. This research project will focus on DNA protamination in human spermatozoa. The purpose of this study is to determine a possible relationship between sperm morphology, viability, concentration and motility, and nuclear protamination of human spermatozoa and secondly to examine if there is a relationship between $CMA_3$ staining, sperm quality, fertilization rate, embryo quality and pregnancy rates of patients during a first treatment cycle.

## 1.1 Infertility treatment

When patients enter the Assisted Reproductive Technology (ART) programme different procedures can be proposed in order to treat their infertility, according to an algorithm which is based on sperm quality (figure 1). If no tubal problem is present intra uterine insemination (IUI) can be suggested as the first line treatment. However, the inseminating motile count (IMC) has to be higher than 1 million good motile spermatozoa [8]. During IUI the semen sample is prepared, which entails that the best, most motile and morphologically normal sperm cells are selected and then injected into the uterus.

The second option is in vitro fertilization (IVF), which will be suggested to the patient when IMC is lower than 1 million good motile spermatozoa and there are at least 4%

morphologically normal cells. During this procedure several oocytes are induced to mature by using hormonal stimulation. Follicle measurements taken during vaginal ultrasound, together with estradiol, luteinizing hormone and progesterone blood values, give information on the number and maturity of oocytes. When follicles have reached a size of 17 to 18 mm, a human chorionic gonadotropin (hCG) injection is given to further mature the oocytes and to induce ovulation and meiotic maturation 34 to 36 hours afterwards. Follicles are punctured one by one (oocyte pick-up (OPU)) and the fluid is evaluated microscopically to identify cumulus oocyte complexes (COC's). COC's are then inseminated with prepared spermatozoa. In the case of IVF treatment there is still a natural selection of sperm cells, because it is less likely that a spermatozoon of bad quality will be able to fertilize an oocyte. IVF can be used for example in case of a tubal factor (damaged or blocked fallopian tube), severe endometriosis and decreased male fertility.

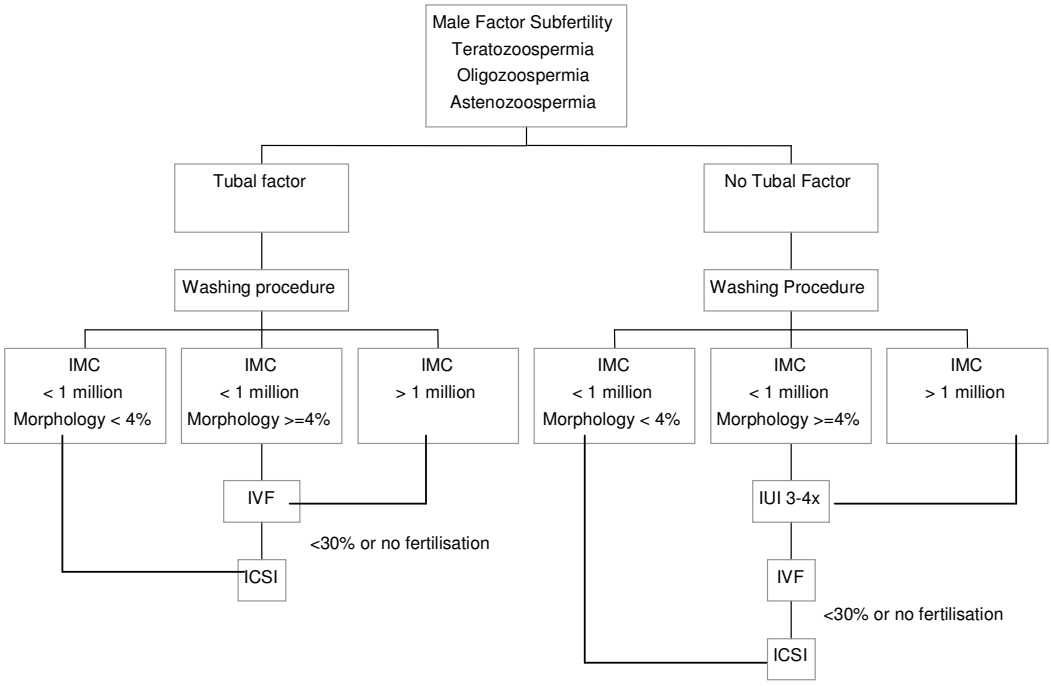


**Figure 1: Algorithm for assessment of male subfertility.** Based on this chart the decision is made to select one specific treatment. (inseminating motile count (IMC) is the total number of good motile spermatozoa after capacitation) [8]

The third technique is intracytoplasmic sperm injection (ICSI). ICSI is indicated when sperm values are as follows: an IMC of less than one million good motile spermatozoa and less than 4% of spermatozoa with a normal morphology and/or 30% or less fertilization after IVF. The collection of the COC's is performed according to the same procedure as described for IVF. When ICSI is applied, one spermatozoon is selected under the microscope at high magnification and injected directly into the oocyte with a

micropipette, hence bypassing sperm cell membrane interaction with the oocyte membrane [9]. Indications for ICSI are the following: fertilization failure in IVF, sperm agglutination, severe oligozoospermia (concentration lower than reference value), cryptozoospermia (sperm found only after centrifugation), severe asthenozoospermia (motility lower than reference value), teratozoospermia (morphology lower than reference value), immunological factors, obstructive and non-obstructive azoospermia (no measurable level of sperm), ejaculatory dysfunction, impaired spermatogenesis or spermiogenesis, oncology, hepatitis C, history of repeated polyspermia after IVF, preimplantation genetic diagnosis, in vitro matured oocytes, … [10].

### 1.2 Spermatogenesis and spermiogenesis

In order for IUI and IVF to be successful mature spermatozoa are to be used. Mature sperm cells are derived from germ cells through a series of complex cellular and genetic transformations in the testes [11]. Spermatogenesis consists of several stages, more specifically: mitotic division of spermatogonia and two meiotic divisions of spermatocytes. Spermiogenesis is the last phase of spermatogenesis and entails the transformation of round spermatids into spermatozoa [11]. During the first meiotic division spermatogonia initially become primary spermatocytes and then turn into two secondary spermatocytes. The second meiotic division results in four haploid round spermatids with unduplicated chromosomes [12]. Further maturation of spermatids into motile spermatozoa will take place during spermiogenesis [11].

Spermiogenesis consists of four stages: Golgi phase, cap phase, acrosomal phase and maturation phase. During the Golgi phase, development of the axonema complex in the tail posterior of spermatozoa takes place. In the cap phase, vesicles formed on the anterior side of cells will transform into acrosomal caps and the nuclear chromatin will also become more condensed. In the acrosomal phase, cytoplasm between the acrosomal cap and anterior cellular membrane will migrate towards the posterior part of the cell. Centrioles will form neck segments of spermatozoa and one of them will synthesize the axonemal complex and eventually produce a tubular complex. Mitochondria will aggregate to surround this tubular complex and compose the mid-piece. The maturation phase is characterized by exclusion of cytoplasm in residual bodies and phagocytosis thereof by Sertoli cells. Developing spermatozoa are then released into the lumen of the seminiferous tubule (spermiation), which marks the end of spermiogenesis. Spermatozoa are transported through the epididymis to the ejaculatory duct while going through maturation [13].

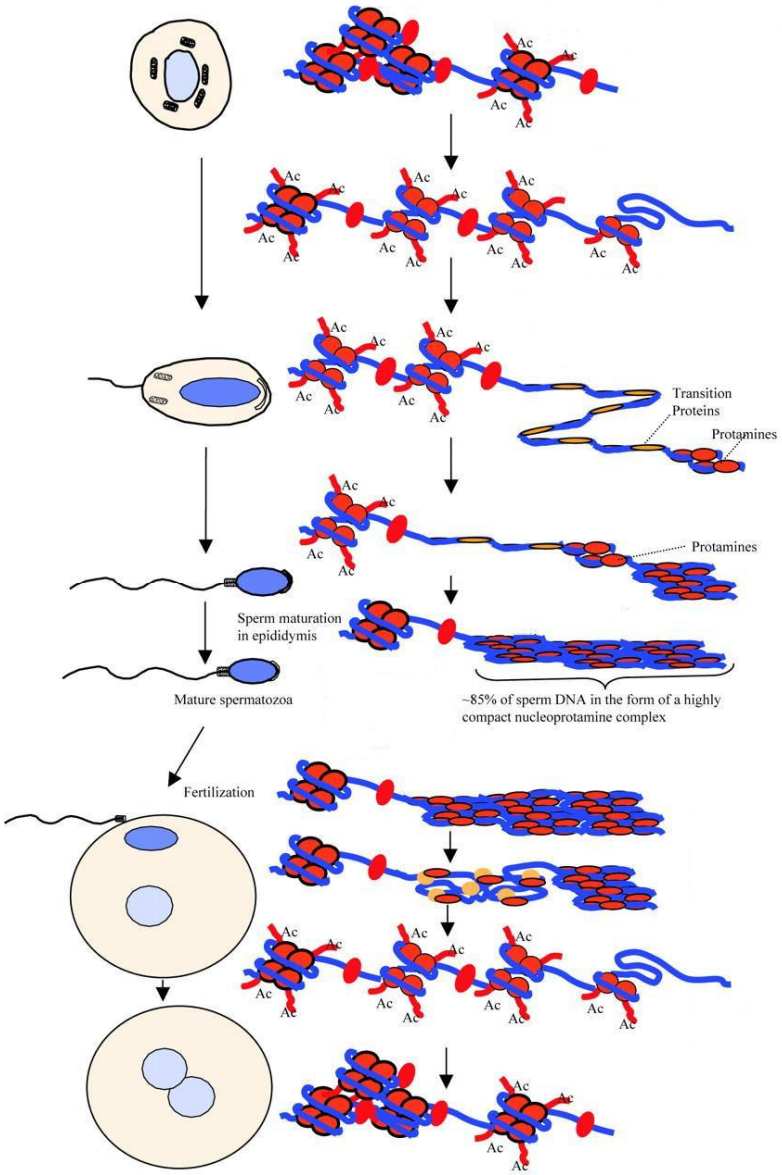


**Figure 2: Changes in chromatin conformation of spermatozoa during spermatogenesis and fertilization.** Nuclear DNA of spermatozoa condenses during sperm maturation through the replacement of histones (red circles) by protamines (red ovals) and after fertilization the DNA decondenses again to its nucleosomal conformation [14]. With courtesy of R. Oliva and journal Human Reproduction Update.

During spermiogenesis, spermatid DNA also undergoes further maturation consisting of DNA packaging, resulting in condensed nuclear DNA [15]. There is an increased transcription and translation of ribonucleic acid (RNA) for protamines and transition proteins during the early stages of spermiogenesis [14]. DNA is packaged with histones, this nucleosomal structure is disassembled and histones are then replaced with transition proteins. Subsequently, transition proteins are removed and protamines will take their place. These processes occur during spermatid elongation [14, 15]. In human sperm, at least two classes of protamines can be found, protamine 1 (P1) and protamine 2 (P2), with a 1:1 ratio present in sperm of fertile men [16]. However, the level of protamine 2

decreases in infertile men, some cases have even been reported in which P2 was completely absent [17], correlating with a decreased ability of sperm cells to penetrate the oocyte [16]. During the stage of spermatid elongation the transcription stops as well and possible DNA strand breaks are repaired. When sperm cells are maturing in the epididymis, protamines bound to DNA crosslink to proceed into a highly compacted genome (figure 2). Compaction of DNA is facilitated by the positive charge of the protamines, which neurtralizes the negative charge of DNA and subsequently the DNA is coiled into a toroidal structure [18]. After protamine synthesis and before deposition on DNA, serine and threonine are phosphorylated, rendering a negative charge to amino acids in protamines. After binding to the DNA, the serine and threonine residues may be dephosphorylated and two cysteines are brought close enough together to allow formation of a disulphide bridge [19]. The high degree of genome compaction leads to an epigenetic silencing and this process is only reverted when the protamines are removed from the genome during early fertilization [20].

## 1.3 Different sperm parameters and ART

Until now the quality of sperm cells is assessed by determining concentration, motility, morphology and viability. However, these routinely used World Health Organization (1999) parameters sometimes fail to predict the fertilization rate correctly, since different fertilization rates have been observed in patients with comparable normal or subnormal semen parameters. Hidden anomalies present in sperm chromatin could be one of the causes for reduced fertilizing potential [2]. An example of such anomalies is defective chromatin protamination.

### *1.3.1 WHO sperm analysis*

Normal morphology is considered to be the parameter which is most related to fertilization in vivo as well as in assisted reproduction [21]. Morphology of a spermatozoon is determined according to the strict criteria as described in the WHO guidelines (1999) [22-24] (supplement 1). Sperm morphology is related to the ability of sperm cells to bind to the zona pellucida and induction of the acrosome reaction [21]. Studies have also found correlations between IVF and motility, concentration and sperm morphology [25-28]. Although initially it was thought that sperm morphology was not important for outcome in ICSI [29], several studies now indicate that there is an association. Authors who use ultra high magnification to assess morphological characteristics of spermatozoa, found that there is an association between implantation and pregnancy rate after ICSI and nuclear morphology of spermatozoa [30, 31]. Other

work has also provided evidence of increased frequencies of aneuploidy in morphologically abnormal spermatozoa [32, 33]. This is an indication for detailed morphological assessment of spermatozoa before applying ICSI.

Sperm concentration is confirmed to be a parameter predicting whether fertilization would take place in IVF, however morphology would be more accurate in doing so [34]. Mean sperm count and motility are also higher in patients who achieved fertilization after IVF [26]. Viability is an important parameter as well, since spermatozoa have to be viable in order to be able to fertilize an oocyte. However, each of these parameters on its own does not predict fertilization and should be used in combination with the other parameters.

### *1.3.2 Functional tests*

As described before, WHO sperm parameters can sometimes fail to predict the fertilization rate correctly and other tests focusing on sperm functionalities, like correct DNA maturation, have been developed. The role of DNA protamination and potential to fertilize in vitro, to deliver good quality embryos and to indicate a healthy pregnancy has to be investigated.

During the last stages of spermiogenesis approximately 85% of nuclear histones in male gametes are replaced by protamines, which are arginine- and cysteine-rich proteins [16, 35, 36]. Histone replacement increases the condensation of sperm chromatin in mammalian cells to up to six times that of a mitotic chromosome [37]. This is facilitated by disulfide bonds which are formed between the cysteine groups of the protamines [36, 38]. Balhorn et al. [19] proposed that protamines bound by the DNA are placed lengthwise in the minor groove. The protamine-DNA complex of one strand would then fit into the major groove of another DNA strand. In this way DNA strands in the sperm nucleus would be packaged side by side [19]. As a result of chromatin packaging sperm cells decrease in size and transcriptional inactivation occurs [39]. Condensation of chromatin may also facilitate sperm motility and protect genetic material from damage [16, 35].

In order to fertilize, sperm chromatin must be able to decondense. To achieve this decondensation disulphide bonds between protamines have to be reduced so that protamines can be removed and the DNA can adapt to its nucleosomal conformation [14]. Poor chromatin packaging like a modification or absence of protamines may contribute to failure of sperm decondensation after IVF and ICSI and can then result in

fertilization failure [2, 40]. Studies have shown that spermatozoa of infertile men more frequently show an abnormal nuclear chromatin organization than those of fertile men [2, 41]. One of the functional tests to analyse protamination in sperm is chromomycin $A_3$ ($CMA_3$) staining. $CMA_3$ is a guanine-cytosine specific fluorochrome which competes with protamines to bind to the DNA [36, 38, 40]. A strong correlation was revealed between $CMA_3$ staining and endogenous nick translation, confirming that $CMA_3$ staining is reliable and strongly correlated with other assays for the evaluation of chromatin [36]. The sensitivity and specificity of this technique have been shown to be comparable to those of aniline blue staining, a technique used in previous studies by different groups [3, 42]. Bizarro et al. [43] indicated that in situ protamination of decondensed mouse and human spermatozoa with salmon protamines, led to a partial recoiling of DNA. Furthermore, this recoiling coincided with a decrease in $CMA_3$ fluorescence [43]. Manicardi et al. [36] demonstrated a strong correlation between abnormal sperm chromatin packaging and DNA strand nicks [36]. A DNA nick is a single strand break in the DNA and endogenous nicks are damaged areas in a DNA strand that occur naturally and can arise for example from replication errors or faulty DNA repair [44]. Practically none of the $CMA_3$ negative spermatozoa showed nicked DNA, whereas $CMA_3$ positive spermatozoa did show DNA nicks [36]. From these studies the authors concluded that $CMA_3$ staining indicates poor chromatin packaging quality and allows indirect visualization of protamine-deficient, nicked and partially denatured DNA [36, 40, 43]. Recent work by Nili et al. [45] indicated that the level of $CMA_3$ positive spermatozoa was higher in subfertile patients in comparison to fertile patients, with the highest level of underprotaminated sperm cells in oligoasthenozoospermic individuals.

Relationships between $CMA_3$ staining and conventional parameters were investigated in different studies. Franken et al. [42] performed a study on a population of men visiting the andrology laboratory. In this study the authors found a significant negative correlation between normal morphology (according to strict criteria) and nuclear maturity. For motility no significant correlation could be detected [42]. Esterhuizen et al. [46] studied a population attending the centre for IVF treatment and detected a highly significant correlation between normal morphology (strict criteria) and $CMA_3$ positivity. Iranpour et al. [41] investigated relationships between $CMA_3$ and several conventional parameters in an IVF population and found a negative correlation for concentration as well as motility. A positive correlation was distinguished for abnormal morphology (Papanicolaou staining) [41]. A study on couples undergoing IVF or ICSI treatment by Tarozzi et al. [18] revealed negative correlations between abnormal protamination and concentration, motility as well as morphology.

Studies carried out to investigate the relationship between $CMA_3$ staining and outcome in ART revealed contradictory results. Iranpour et al. [41] reported a negative correlation between $CMA_3$ fluorescence and fertilization ability for patients treated by IVF, whereas Bianchi et al. [2] indicated that there was no association between $CMA_3$ positivity and IVF treatment. Sperm decondensation after ICSI was assessed as well. Patients with higher $CMA_3$ positivity had a higher number of unfertilised oocytes containing condensed spermatozoa [40]. Tarozzi et al. [18] found a relationship between sperm cell protamination and fertilization and pregnancy rates in IVF, but not in ICSI. Hammadeh et al. [5] failed to demonstrate a relationship between chromatin condensation assessed by Aniline Blue staining and the fertilization potential, cleavage rate and pregnancy rate after ICSI [5]. A negative correlation was revealed between DNA damage, measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), and the fertilization rate after IVF, but not after ICSI. The number of DNA abnormalities were also significantly lower in patients who achieved pregnancy [47]. Another study by Esterhuizen et al. [46] indicated a negative correlation between $CMA_3$ values and IVF results. Nijs et al. [48] found in a recent study that $CMA_3$ had a predictive value for obtaining a pregnancy and having a healthy baby in IVF and combo IVF/ICSI cycles. During combo IVF/ICSI sibling oocytes will be inseminated by IVF and ICSI. However these correlations were only found when two parameters (% $CMA_3$ and % high DNA stainability (HDS) in SCSA) were combined in logistic regression. Spermatozoa with HDS have immature DNA with unprocessed nuclear proteins and/or poorly condensed chromatin [48].

### 1.4 Aim of the study

The overall aim of this prospective cohort pilot study is to determine if there is a relationship between sperm morphology, viability, concentration and motility, and nuclear maturity of human spermatozoa as demonstrated by $CMA_3$ staining. Secondly the purpose is to investigate if there is a relationship between $CMA_3$ staining, sperm quality, fertilization rate, embryo quality and pregnancy rates of patients after a first IUI, IVF or ICSI. It is hypothesized that there will be a correlation between at least one of the conventional parameters and DNA protamination and that there will be a correlation between DNA protamination, sperm quality and the fertilization and pregnancy rates of the patients. By conducting this study, $CMA_3$ staining can possibly become one of the routine tests for semen evaluation. Thus, a better assessment of the quality of sperm cells used for fertilization will be possible and there can be better indications for the type of fertility treatment that should be selected.

# 2 Materials and methods

In the materials and methods section, inclusion and exclusion criteria for this study will be listed. Evaluation of semen by using WHO tests and $CMA_3$ staining will be explained. Also sperm preparation, IUI, IVF and ICSI procedures will be described. Evaluation of fertilization and pregnancy rates will be discussed and an overview will be given of the main statistical analyses that were applied. Finally, the entire processing of a fresh semen sample will be summarized in a flowchart (figure 3).

## 2.1 Patients

All patients entering the ART programme at the Genk Institute for Fertility Technology for a first IUI, IVF or ICSI treatment were included in the study. The study was approved by the local ethical committee and all patients signed an informed consent form. Treatment mode itself was determined by the gynaecologist according to the algorithm in figure 1. Female partners were younger than 37 years and only cycles with ejaculated spermatozoa were included in the study. Exclusion criteria for this study were: previous ART attempt(s), severe oligozoospermia, asthenozoospermia and teratozoospermia and the use of testicular sperm cells. On the day of treatment a semen sample was produced after two days of abstinence. Patients did not report fever or other illnesses for a period of 8 weeks preceding the study.

## 2.2 Semen analysis according to WHO guidelines

Analysis of semen samples was performed within 1 hour of production and after liquefaction. Samples of the IVF/ICSI group were collected for investigation in this study 5-7 days before OPU and on the day of OPU. Samples of the IUI group were collected on the day of insemination. Sperm motility, concentration and viability were evaluated according to WHO criteria and morphology was assessed according to strict criteria (WHO, 1999) [24]. All parameters were determined by using raw, neat semen samples.

### *2.2.1 Motility*

Sperm motility was evaluated by grading the progressive movement of spermatozoa. In a wet preparation at least two hundred spermatozoa were graded in minimally 5 randomly selected microscopic fields at 400x magnification under a microscope with phase contrast. Spermatozoa were evaluated in two separate wet preparations each

containing 14.5 µl of semen. Before assessing motility, the preparation should be left to rest for one minute on a 37°C heated microscope table. Motility was divided into four categories from grade A until D. With grade A comprising rapid progressive, directional spermatozoa (> 25 µm/s); grade B slow progressive, non-directional and the fast progressive, non-directional spermatozoa (5-25 µm/s); grade C non-progressive spermatozoa (< 5 µm/s) and grade D immotile spermatozoa (0 µm/s). The WHO reference value for semen normality is ≥ 50% motile spermatozoa (grade A + B) or ≥ 25% grade A within 60 minutes of ejaculation. According to Ombelet et al. [49] a more realistic reference value would be ≥ 45% grade A+B motile spermatozoa or ≥ 8% grade A spermatozoa. Good motile spermatozoa consist of grade A and grade B spermatozoa and total motility is calculated as the sum of grade A, B and C sperm cells [22, 48].

### *2.2.2 Concentration*

Sperm concentration or sperm count is the number of spermatozoa that are present in 1 ml of sample. Concentration was assessed by counting cells in a haemocytometer and this after homogenisation and dilution. First an estimation of the number of cells was made in a wet preparation of 14.5 µl of sample by looking at a magnification of 400x. Then, based on this estimation the right dilution was determined (1:5, 1:10, 1:20 or 1:50) and water was added to dilute 50 µl of homogenized sample (table 1). The sample was pipetted by use of a positive displacement pipette. By adding water, sperm cells become immobile. The haemocytometer was filled on two sides with two separately made dilutions. Following this, the haemocytometer was placed in a moist chamber during 5 min. This allowed the cells to settle and avoided dehydration. A maximum of twenty-five blocks from the central grid should be counted on each side of the chamber on 400x magnification and at least 200 cells should be counted on each side of the haemocytometer. Depending on sample dilution different factors were used to calculate the concentration (table 1). Only intact spermatozoa were counted, so heads without a tail, tails alone or pinheads were excluded. The WHO reference value for semen normality is ≥ $20.10^6$ spermatozoa/ml. The reference value according to Ombelet et al. [49] is > $34.10^6$ spermatozoa/ml.

**Table 1: Determining the dilution of the sample and factors to calculate concentrations of spermatozoa in $10^6$/ml.**
* the number of spermatozoa counted in one field at 400x magnification in a wet preparation **number of counted blocks

| *# spermatozoa per field** | *Dilution* | *Factor to calculate concentration* | | |
|---|---|---|---|---|
| | | *5*** | *10*** | *25*** |
| <15 | 1:05 | 0.25 | 0.125 | 0.05 |
| 15-40 | 1:10 | 0.5 | 0.25 | 0.1 |
| 40-200 | 1:20 | 1 | 0.5 | 0.2 |
| >200 | 1:50 | 2.5 | 1.25 | 0.5 |

*2.2.3 Viability*

By assessing the viability of sperm cells a percentage of living cells is determined. Living cells are necessary to achieve successful fertilization. Whether a sample contains viable spermatozoa can be determined by eosin dye exclusion. A distinction can be made between dead and viable sperm cells, because dead sperm cells with a damaged plasma membrane will take up the eosin, whereas viable spermatozoa will not show any staining. The semen sample was homogenized, 50 µl of sample was collected with a positive displacement pipette, eosin solution was added in a 1:1 ratio and this was vortexed. After adding eosin, the solution should rest for 2 min. Then two preparations were made with each 10 µl of the solution. For each preparation 100 cells were counted on a 400x magnification. The WHO reference value for semen normality is ≥ 50% cells excluding the dye [22]. The reference value for viability according to Ombelet is > 40% viable cells.

*2.2.4 Morphology*

Sperm morphology was determined by individual scoring of morphological characteristics of spermatozoa. In order to assess the morphology of samples the modified Papanicolaou staining was applied [22, 23]. This staining allows to distinguish between acrosomal and post-acrosomal regions of the head segment, cytoplasmic droplets, middle segment and tail and gives a clear difference between acidic and basic cell components. Acrosomal regions of head segments were stained pale blue, whereas post-acrosomal regions of head segments were dark blue. Midpieces were stained red, tails were stained blue or reddish and cytoplasmic droplets were stained green. 20 µl sample was spread on a glass slide and allowed to air-dry. Then slides were stained by using the Papanicolaou method. At least two smears were used and minimally 100 cells were evaluated per slide on 1000x magnification with immersion oil. A differentiation was made between spermatozoa with a normal and abnormal morphology, according to strict criteria [24]. This differentiation was based on head defects, neck and midpiece defects, tail defects and cytoplasmic droplets (supplement 1). The reference value for semen normality according to the WHO is > 14% normal spermatozoa [22]. Reference values for morphology as studied by Ombelet et al. [49] are: < 5% normal spermatozoa is severe teratozoospermia, ≥ 5% and < 10% normal spermatozoa is moderate teratozoospermia and ≥ 10% normal spermatozoa is normal morphology.

### 2.3 $CMA_3$ staining

200 µl of the semen sample was used for $CMA_3$ staining. The sample was washed twice in Dulbecco's $Ca^{2+}$-$Mg^{2+}$-free PBS (Sigma-Aldrich), with a ratio of two parts PBS to one part of semen. The sample was centrifuged for 10 min at 1200 rotations per minute (rpm). The sediment of washed spermatozoa was fixated in a methanol/acetic acid mixture (3:1) (VWR; Sigma-Aldrich) for 5 min at 4°C with a volume dependent on the concentration and solvability of the sample (concentration $>30x10^6$ spermatozoa/ml: 200 µl, $<30x10^6$ spermatozoa/ml: 100 µl) . After homogenization the spermatozoa were spread on two glass slides for each patient, by letting two or three drops (depending on the concentration) fall on the slide and tilting the slide in order for the sperm cells to spread across the slide. Slides were air-dried and stained. Actual staining could be performed with 100 µl $CMA_3$ solution during 20 min at room temperature (Sigma-Aldrich, 0.25 mg/ml in McIlvaine buffer, pH 7.0, containing 10 mM $MgCl_2$ (supplement 2)). Slides were rinsed with PBS, air-dried and covered with a glass cover slip by using a 1:1 solution of PBS and glycerol [36, 42]. Slides were kept overnight at 4°C and were examined the next day by fluorescence microscopy at 390-490 nm wavelength (Leitz, Laborlux D, Wetzlar, Germany). Spermatozoa with protaminated DNA stained dull yellow, whereas cells with underprotaminated DNA stained bright yellow. Five hundred cells were examined in an all-or-nothing approach on a magnification of 500x by using immersion oil. The reference value for good protamination as assessed by $CMA_3$ staining is ≤ 30% positive spermatozoa [46, 50].

### 2.4 Morphology at time of $CMA_3$ evaluation

Each sperm cell at the time of $CMA_3$ fluorescence evaluation was also scored according to the morphology of the head segment. Using bright light microscopy, a distinction could be made between a normal, macrocephalic or amorphous head segment. A clear distinction needs to be made between the two different types of morphology scoring. Morphology scoring after Papanicolaou staining (strict criteria) is more detailed and gives an evaluation of the head, midpiece and tail defects. Morphology scoring at time of $CMA_3$ evaluation only considers the sperm head. Spermatozoa were categorized as normal when the head has an oval shape and is between 4.0 µm and 5.5 µm in length and between 2.5 µm and 3.5 µm in width. Macrocephalic spermatozoa have a larger shaped head compared to the normal sperm cells. Sperm heads are classified as amorphous when they display an irregular shape, but not larger than normal, vacuoles, acrosomal abnormalities, cytoplasmic remnants, ...

## 2.5 Sperm preparation

For IUI, IVF and ICSI semen samples need to be prepared in order to select the most motile and normal sperm cells. Volume, concentration, motility, viability and morphology were determined according to the WHO guidelines. Following this, samples were prepared for use in IUI, IVF or ICSI. Preparation started within 1 hour after ejaculation and after sample homogenisation. A density gradient centrifugation was performed on three layers (Pure Sperm 100 (Nidacon); 90%, 70%, 40%) for 20 minutes at 1700-1900 rpm as described by Nijs [23]. Sperm cells in the 90% layer were washed twice for 10 min at 1700-1900 rpm in Earle's balanced salt solution (EBSS) (Sigma-Aldrich) supplemented with 5% human serum albumin (HSA) (Rode Kruis). This washing step was repeated a second time. The supernatatant was removed, the pellet was resuspended in EBSS and samples were equilibrated in 1ml EBSS with 5% HSA in a $CO_2$ incubator at 36.5°C for at least 15 minutes [48].

## 2.6 IUI

After sperm preparation 1 ml of sperm cell solution was obtained. Prepared cells were then aspirated in an IUI catheter and injected in the uterus of the patient.

## 2.7 Oocyte culture and embryo culture

During oocyte pick-up oocytes were collected and washed in G-MOPS (Vitrolife) supplemented with 0.5% HSA (Rode Kruis). Oocyte and embryo culture was carried out in test tubes with sequential media (Sage media or with Vitrolife media), all supplemented with 0.5% HSA (Rode Kruis). All media were gassed with 5% $CO_2$, 5% $O_2$ in $N_2$, tubes were tightly capped and placed at 37°C. Media were prepared 24h in advance to allow equilibration. After each daily morphological evaluation, media were changed. Embryo culture was performed until day 5 post fertilization.

## 2.8 IVF and ICSI

In IVF 100,000 grade A motile spermatozoa per ml or 500,000 grade A and B motile spermatozoa per ml were used for insemination of 5 to 8 COC's. In ICSI spermatozoa were placed in the middle of a medium drop under oil and were allowed to swim out to the edge of the drop for a period of 5 to 10 minutes. Then, the most motile spermatozoa were collected from the edge of the droplet by using an ICSI pipette and placed in a

polyvinylpyrrolidone (PVP) droplet (Vitrolife or Sage). Following this, one spermatozoon with the best morphological features was selected at high magnification (640x). This sperm cell was subsequently immobilised and injected into the oocyte. Only metaphase II oocytes were used for ICSI [48].

### 2.9 Evaluation of fertilization and embryo transfer

Sixteen to eighteen hours after IVF or ICSI fertilization was evaluated. Mean fertilization rate was determined by calculating the number of fertilized oocytes, with two pronuclei (one female and one male), per number of metaphase II oocytes. Embryo quality was assessed according to the grading system of Staessen et al. [51]. This grading system takes number of cells, speed of development, percentage fragmentation and percentage of granularity into account, among other factors important in embryo development. Embryos were transferred with an embryo transfer catheter into the uterus on day 2, 3, 4 or 5 of culture. In a first IVF and ICSI treatment, a single embryo is transferred. Embryo quality (%) is defined as the number of top quality embryos (grade A or B) on the day of transfer per total number of fertilized oocytes obtained.

### 2.10 Evaluation of pregnancy

Pregnancy rate was assessed by measuring serum β hCG levels, on day 12 and day 14 after embryo transfer or insemination. For the second test, β hCG concentration should be at least double the amount of hCG that was measured during the first test. Pregnancy rate is defined as the ratio of the number of pregnancies to the number of embryo transfers. Clinical pregnancy rates was determined six weeks after embryo transfer or insemination by confirming the presence of a gestational sac with positive heartbeat during ultrasound.

### 2.11 Statistical analysis

Correlations between concentration, motility, viability, morphology and $CMA_3$ values were calculated by using the Pearson's correlation coefficient. Pearson's correlation coefficient analysis was also used to indicate correlations between conventional or functional parameters. Linear regression is used to evaluate the differences between the distribution of parameters.

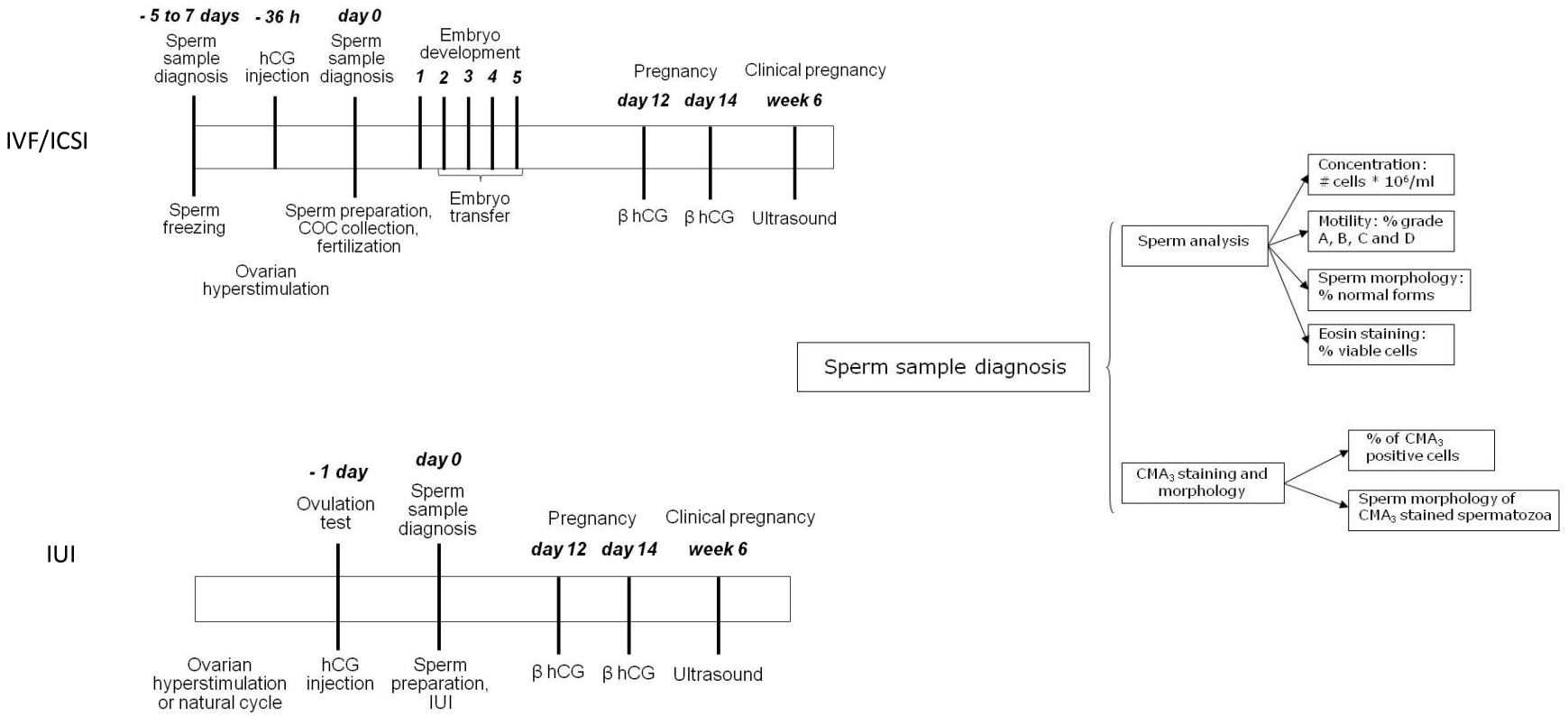


**Figure 3: Analyses prior to and post treatment.** In the flowchart all the different tests are listed which are carried out on the fresh semen sample.

# 3 Results

This prospective study covered a period of 4 months (10 march 2009 – 10 july 2009). Fifty-two patients were enrolled in the study. Drop out was noted for the following patients: 4 patients had previous ART attempts, 2 patients had a poor quality sample on the day of oocyte pick-up, 3 patients stopped treatment after stimulation failure, 1 female patient was older than 37 years and for two patients the period of 2 days abstinence was not respected. So the total study population consists of 40 patients: 22 in the IUI group and 18 in the IVF/ICSI group. The IVF/ICSI group consists of 2 patients undergoing IVF, 7 ICSI and 9 combo IVF/ICSI.

## 3.1 Demographic data

Demographic details of the patients are summarized in table 2. Mean values of semen parameters for all patients studied are: concentration 63.65 x $10^6$ spermatozoa/ml, 51.7% good motility (grade A+B), 55.5% total motility (grade A+B+C), 72.8% viable cells, 4.4% morphologically normal cells (strict criteria) and 4.7% normal morphology (at time of $CMA_3$). For $CMA_3$ staining a mean value of 39.2% positive sperm cells is found. In the IUI group 38.3% $CMA_3$ positive sperm cells are found, for IVF/ICSI 5-7 days before OPU 41.5% and for IVF/ICSI on day of OPU 40.3% $CMA_3$ positive sperm cells are detected (figure 4 and 5).

**Table 2: Demographic data of overall patient population and subgroups.** Mean values are shown in brackets with standard deviation, minimum and maximum respectively. * for one patient no data could be obtained 5-7 days before OPU

| | ***Overall*** | ***IUI*** | ***IVF/ICSI 5-7 days before OPU*** | ***IVF/ICSI day of OPU*** |
|---|---|---|---|---|
| **Number of patients** | **40** | **22** | **17*** | **18** |
| **Age female (in years)** | **30.3** (±3.1; 24-36) | **29.3** (±3.3; 24-35) | **31.6** (±2.6; 28-36) | **31.5** (±2.5; 28-36) |
| **Age male (in years)** | **33.6** (±5.2; 24-47) | **32.8** (±4.6; 24-46) | **34.9** (±5.8; 27-47) | **34.6** (±5.8; 27-47) |
| **Concentration ($10^6$ spermatozoa/ml)** | **63.65** (±50.67; 4-220) | **73.27** (±48.58; 14.5-200) | **63.64** (±57.64; 0.8-189) | **51.89** (±52.04; 4-220) |
| **Good motile cells (% grade A+B)** | **51.7** (±14.4; 26-72) | **54.3** (±12.8; 27-72) | **51.4** (±16.8; 5-74) | **48.6** (±15.8; 26-72) |
| **Total motile cells (% grade A+B+C)** | **55.5** (±14.1; 30-83) | **57.8** (±12.7; 30-78) | **54.8** (±15.7; 14-78) | **52.6** (±15.5; 30-83) |
| **Viability (% viable sperm cells)** | **72.8** (±10.7; 40.5-88.5) | **73.9** (±10.6; 52-88.5) | **67.6** (±14.6, 30.5- 82) | **71.3** (±10.9; 40.5-86.5) |
| **Morphology (% normal spermatozoa, strict criteria)** | **4.4** (±3.9; 0-15.5) | **5.0** (±3.9; 0.5-15.5) | **4.9** (±3.7; 1-12) | **3.8** (±3.9; 0-13) |
| **$CMA_3$ positive spermatozoa (%)** | **39.2** (±17.5; 14.2-74.8) | **38.3** (±17.1; 14.2-74.8) | **41.5** (±21.8; 14.6-82.2) | **40.3** (±18.4; 18.4-74.2) |
| **Sperm head morphology at time of $CMA_3$ (% normal head morphology)** | **4.7** (±3.3; 0.4-12.2) | **5.5** (±3.4; 0.4-12.2) | **4.4** (±3.4; 1-12.2) | **3.9** (±3.0; 0.6-11) |

No significant differences could be found between the total number of $CMA_3$ positive cells in the IUI group, the IVF/ICSI group 5-7 days before OPU and the IVF/ICSI group on the

day of OPU (figure 4) (IUI-IVF/ICSI 5-7 days before OPU, p=0.95; IUI-IVF/ICSI day of OPU, p=0.72; IVF/ICSI 5-7 days before OPU-IVF/ICSI day of OPU, p=0.78).

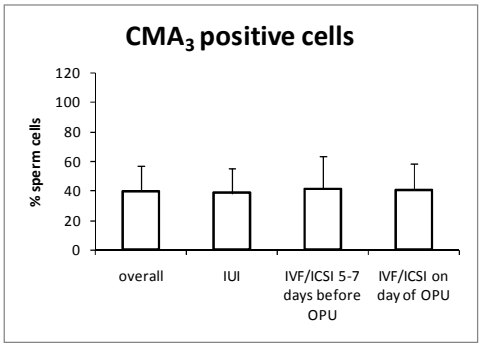


**Figure 4: Graphical representation of the number of $CMA_3$ positive cells for the overall population and subgroups.** Data are represented as mean with standard deviation.

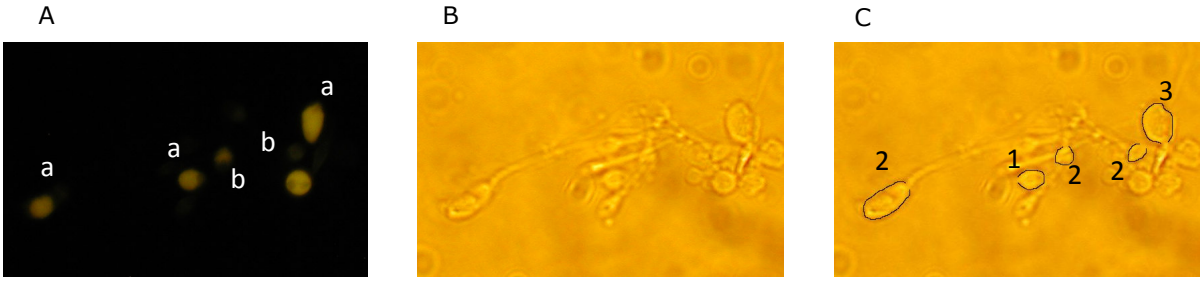


**Figure 5: Sperm analysis.** Microscopic analysis at 500x magnification. **A:** $CMA_3$ staining (a: bright yellow cells: $CMA_3$ positive, poor protamination; b: dull yellow cells: $CMA_3$ negative, good protamination), **B:** Bright light: sperm head morphology scoring, **C:** Sperm head morphology; 1: normal sperm head, 2: amorphous sperm head, 3: macrocephalic sperm head

## 3.2 $CMA_3$ and morphology

Sperm head morphology of fluorescent and non-fluorescent sperm cells was scored simultaneously with $CMA_3$ analysis revealing the following results for the overall patient group: of all spermatozoa scored 4.7% had normal, 0.7% macrocephalic and 94.6% amorphous head morphology (table 3). These results are displayed in figure 6.

**Table 3: Sperm head morphology of semen samples at time of $CMA_3$ scoring.** Mean values are shown in brackets with standard deviation, minimum and maximum respectively. * for one patient no data could be obtained 5-7 days before OPU

| | ***Overall*** | ***IUI*** | ***IVF/ICSI 5-7 days before OPU*** | ***IVF/ICSI day of OPU*** |
|---|---|---|---|---|
| **Number of patients** | **40** | **22** | **17*** | **18** |
| **Normal sperm head morphology (%)** | **4.7** (±3.3; 0.4-12.2) | **5.5** (±3.4; 0.4-12.2) | **4.4** (±3.4; 1-12.2) | **3.9** (±3.0; 0.6-11) |
| **Macrocephalic sperm head morphology (%)** | **0.7** (±0.9; 0-4.2) | **0.8** (±1.2; 0-4.2) | **0.6** (±0.6; 0-1.8) | **0.6** (±0.4; 0-1.4) |
| **Amorphous sperm head morphology (%)** | **94.6** (±3.8; 83.6-99.6) | **93.7** (±4.3, 83.6-99.6) | **95.0** (±3.2; 87.6-98.6) | **99.6** (±3.0; 88.2-98.8) |

No significant differences can be detected for the amount of spermatozoa with normal head morphology between the IUI group and the IVF/ICSI group at both time points (T1 and T2) (IUI-IVF/ICSI T1, p=0.50; IUI-IVF/ICSI T2, p=0.12). Macrocephalic and amorphous sperm head morphology are also not significantly different for the IUI and the two IVF/ICSI subgroups (macrocephalic: IUI-IVF/ICSI T1, p=0.0.53; IUI-IVF/ICSI T2, p=0.45; amorphous: IUI-IVF/ICSI T1, p=0.29; IUI-IVF/ICSI T2, p=0.13).

Distribution of morphology was evaluated within groups. For the overall group significant differences were found between the normal and macrocephalic sperm heads ($p < 0.0001$), between the normal and amorphous sperm heads ($p < 0.0001$) as well as between the macrocephalic and amorphous sperm heads ($p < 0.0001$). The results were identical for the IUI group and the IVF/ICSI group at both time points.

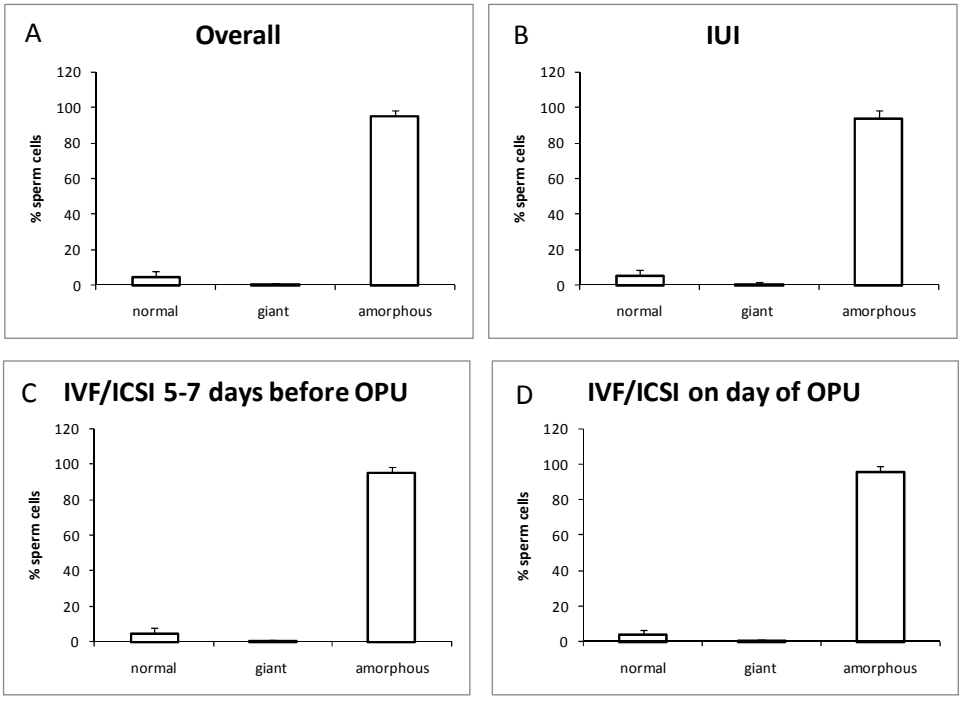


**Figure 6: Graphical representation of sperm head morphology scoring.** Data are represented as mean with standard deviation. The first bar represents the mean percentage of spermatozoa with normal head morphology, the second bar is the mean percentage of spermatozoa with macrocephalic head morphology and the third bar is the mean percentage of spermatozoa with amorphous head morphology. **A:** Mean values for the overall study population, **B:** Mean values for the IUI group, **C:** Mean values for the IVF/ICSI group 5-7 days before OPU, **D:** Mean values for the IVF/ICSI group on the day of OPU

Table 4 summarizes the distribution of sperm head morphology for $CMA_3$ positive cells. For the overall patient population, of the $CMA_3$ positive sperm cells 3.0% have normal head morphology, 1.8% are macrocephalic spermatozoa and 95.2% have amorphous head morphology. For IUI the $CMA_3$ positive spermatozoa consist of 3.8% sperm cells with normal head morphology, 1.9% macrocephalic spermatozoa and 94.3% amorphous

spermatozoa. For IVF/ICSI on the day of OPU $CMA_3$ positive spermatozoa contain 2.1% normal, 1.6% macrocephalic and 96.3% amorphous head morphology. The results of these analyses are represented in figure 7.

**Table 4: Sperm head morphology scoring at time of $CMA_3$ evaluation for $CMA_3$ positive spermatozoa.** Mean values are shown of the morphology scoring for the $CMA_3$ positive spermatozoa, with in brackets standard deviation, minimum and maximum respectively. The sum of % $CMA_3$ positive sperm heads for the three classes of morphology is 100%. * for one patient no data could be obtained 5-7 days before OPU

| *Sperm head morphological scoring at time of $CMA_3$* | *Overall* | *IUI* | *IVF/ICSI 5-7 days before OPU* | *IVF/ICSI day of OPU* |
|---|---|---|---|---|
| **Number of patients** | **40** | **22** | **17*** | **18** |
| **positive normal (%)** | **3.0** (±3.4; 0-12.9) | **3.8** (±3.9; 0-12.9) | **2.3** (±2.9; 0-11.7) | **2.1** (±2.5; 0-8.5) |
| **positive macrocephalic (%)** | **1.8** (±1.6; 0-6.8) | **1.9** (±1.6; 0-5.4) | **1.4** (±1.1; 0-3.1) | **1.6** (±1.6; 0-6.8) |
| **positive amorphous (%)** | **95.2** (±4.7; 83.3-100) | **94.3** (±5.3; 83.3-100) | **96.4** (±3.3; 85.2-99.4) | **96.3** (±3.8; 85.7-100) |

For the $CMA_3$ positive spermatozoa no significant differences can be detected for the distribution of the types of sperm heads that are fluorescent between all three subgroups (normal: IUI-IVF/ICSI T1, p=0.18; IUI-IVF/ICSI T2, p=0.10; macrocephalic: IUI-IVF/ICSI T1, p=0.19; IUI-IVF/ICSI T2, p=0.54; amorphous: IUI-IVF/ICSI T1, p=0.14; IUI-IVF/ICSI T2, p=0.17).

However, significant differences can be detected in the overall group between the $CMA_3$ positive normal and macrocephalic sperm heads (p=0.04), between normal and amorphous sperm heads ($p < 0.0001$) and between macrocephalic and amorphous sperm heads ($p < 0.0001$). The results are identical for the IUI group (normal-macorcepholic, p=0.04; normal-amorphous, $p < 0.0001$; macrocephalic-amorphous, $p < 0.0001$). For the IVF/ICSI patients no significant differences can be found between the normal and macrocephalic sperm heads on both time points (T1: p=0.21, T2: p=0.51). For these group a significant difference was found between the normal and amorphous sperm heads ($p < 0.0001$) as well as the macrocephalic and amorphous sperm heads ($p < 0.0001$).

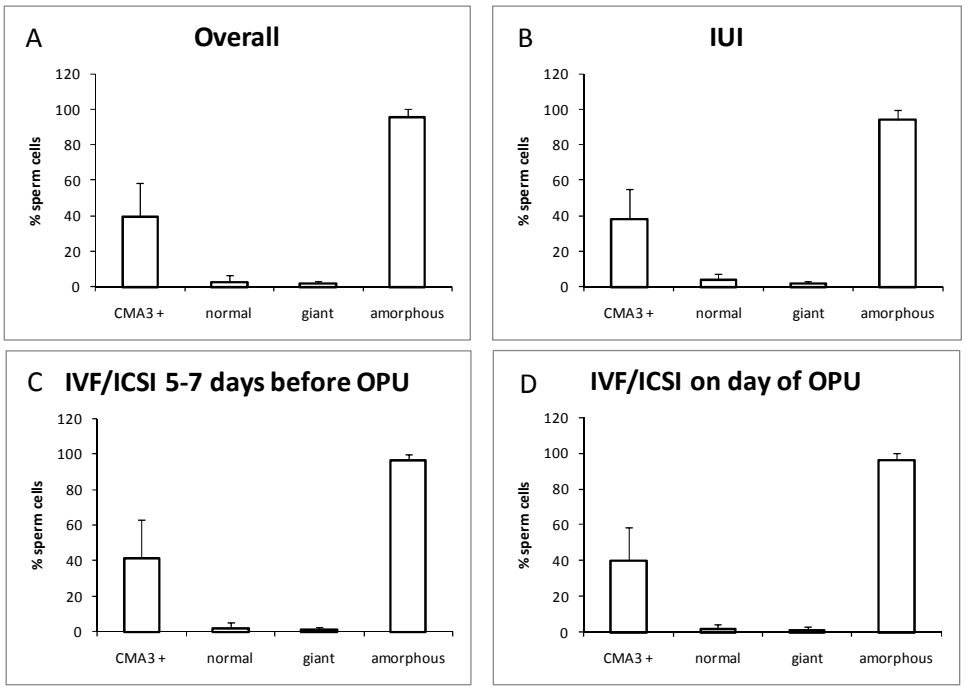


**Figure 7: Graphical representation of the number of $CMA_3$ positive cells, comparing sperm head morphology scoring.** Data are represented as mean with standard deviation. The first bar represents the percentage of $CMA_3$ positive cells, the second bar is the percentage of spermatozoa with normal head morphology out of the $CMA_3$ positive cells, the third bar is the percentage of spermatozoa with macrocephalic head morphology out of the $CMA_3$ positive cells and the fourth bar is the percentage of spermatozoa with amorphous head morphology out of the $CMA_3$ positive cells. **A:** Mean values for the overall study population, **B:** Mean values for the IUI group, **C:** Mean values for the IVF/ICSI group 5-7 days before OPU, **D:** Mean values for the IVF/ICSI group on the day of OPU

After observations of fluorescence and sperm head morphology in the overall study population 17.0% spermatozoa with a normal head shape fluoresced, whereas 92.7% of macrocephalic sperm cells and 39.8% amorphous spermatozoa displayed fluorescence (table 5 and figure 8). Similar fluorescence patterns are observed for the IUI and IVF/ICSI subgroups.

**Table 5: Summary of the number of fluorescent normal, macrocephalic and amorphous sperm heads in all groups.** Mean values are shown in brackets with standard deviation, minimum and maximum respectively. * for one patient no data could be obtained 5-7 days before OPU

| | ***Overall*** | ***IUI*** | ***IVF/ICSI 5-7 days before OPU*** | ***IVF/ICSI day of OPU*** |
|---|---|---|---|---|
| **Number of patients** | **40** | **22** | **17*** | **18** |
| **Normal sperm head morphology (%)** | **17.0** (±15.5; 0-62.5) | **13.6** (±15.0; 0-59) | **23.3** (±27.5; 0-80) | **20.2** (±15.7; 0-62.5) |
| **Macrocephalic sperm head morphology (%)** | **92.7** (±17.3; 33.3-100) | **90.0** (±20.8; 33.3-100) | **92.0** (±14.9; 57.1-100) | **95.1** (±14; 50-100) |
| **Amorphous sperm head morphology (%)** | **39.8** (±18.2; 14.9-76.1) | **37.9** (±18.0; 14.9-76.1) | **43.1** (±21.9; 15.3-82.3) | **41.5** (±18.7; 19.3-74.6) |

No significant differences could be detected for the number of fluorescent normal, macrocephalic and amorphous sperm heads between the IUI subgroup and the IVF/ICSI group 5-7 days before OPU as well as on the day of OPU (normal: IUI-IVF/ICSI T1,

p=0.20; IUI-IVF/ICSI T2, p=0.20;; macrocephalic: IUI-IVF/ICSI T1, p=0.76; IUI-IVF/ICSI T2, p=0.41; amorphous: IUI-IVF/ICSI T1, p=0.45; IUI-IVF/ICSI T2, p=0.56).

Distribution of the number of fluorescent normal, macrocephalic and amorphous sperm heads was evaluated within groups. In the overall group significant differences are detected between the normal and macrocephalic sperm heads ($p < 0.0001$), normal and amorphous sperm heads ($p < 0.0001$) and macrocephalic and amorphous sperm heads ($p < 0.0001$). The results for the IUI group are identical. For the IVF/ICSI group the results are: $p < 0.0001$ on T1 and T2; $p=0.02$ on T1 and $p=0.0002$ on T2 and $p < 0.0001$ on T1, respectively.

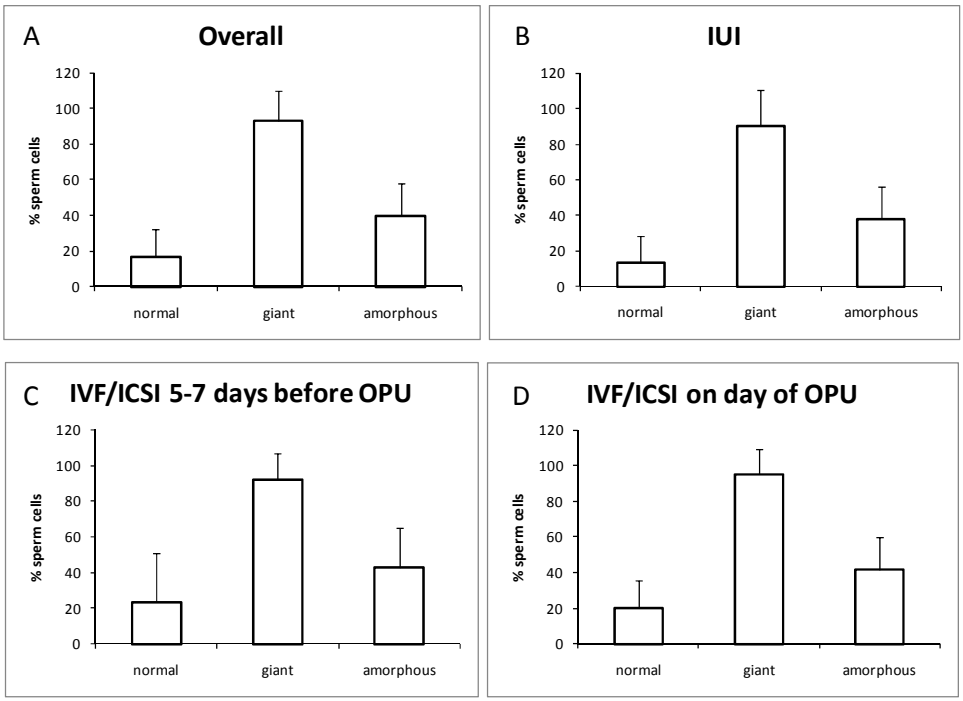


**Figure 8: Graphical representation of the number of fluorescent normal, macrocephalic and amorphous sperm heads.** Data are represented as mean with standard deviation. The first bar represents the mean percentage of fluorescent spermatozoa with normal head morphology, the second bar the mean percentage of fluorescent spermatozoa with macrocephalic head morphology and the third bar the mean percentage of fluorescent spermatozoa with amorphous head morphology. **A:** Mean values for the overall study population, **B:** Mean values for the IUI group, **C:** Mean values for the IVF/ICSI group 5-7 days before OPU, **D:** Mean values for the IVF/ICSI group on the day of OPU

### 3.3 Sperm parameters at different time points

For IVF/ICSI patients, analysis of the four different sperm parameters, $CMA_3$ staining and sperm head morphology scoring was performed at two different time points: 5-7 days before OPU and on the day of OPU.

Table 6 lists the Pearson's correlation coefficients for the two different time points. All parameters on time point 1 demonstrate a significant positive correlation with the respective parameters on time point 2: concentration (R=0.87166, p=<0.0001), good motility (R=0.70657, p=0.002), total motility (R=0.74342, p=0.0006), viability (R=0.63207, p=0.01), morphology (R=0.58301, p=0.02), $CMA_3$ positive spermatozoa (R=0.88228, p=<0.0001) and sperm head morphology at time of $CMA_3$ (R=0.76309, p=0.001).

**Table 6: Comparison of parameters for the IVF/ICSI group 5-7 days before OPU (Time point 1) and on the day of OPU (Time point 2).** Pearson's correlation coefficients are listed as R-values and level of significance as p-values. Correlation is significant at [a] 0.05 level [b] 0.001 level

| *Time point 1* \ *Time point 2* | | *Concentration* | *Good motile* | *Total motile* | *Viability* | *Morphology (strict criteria)* | *$CMA_3$ positive spermatozoa* | *Sperm head morphology at time of $CMA_3$* |
|---|---|---|---|---|---|---|---|---|
| **Concentration ($10^6$ spermatozoa/ml)** | R | 0.87166[b] | 0.41239 | 0.36371 | 0.45418 | 0.51199[a] | -0.17582 | 0.09144 |
| | p | <0.0001 | 0.10 | 0.15 | 0.08 | 0.04 | 0.50 | 0.73 |
| **Good motile cells (% grade A+B)** | R | 0.29859 | 0.70657[a] | 0.72493[a] | 0.47800 | 0.16435 | -0.24809 | 0.23406 |
| | p | 0.24 | 0.002 | 0.001 | 0.06 | 0.53 | 0.34 | 0.37 |
| **Total motile cells (% grade A+B+C)** | R | 0.28443 | 0.72064[a] | 0.74342[a] | 0.56928[a] | 0.14930 | -0.26223 | 0.30695 |
| | p | 0.26 | 0.001 | 0.0006 | 0.02 | 0.57 | 0.31 | 0.23 |
| **Viability (% viable sperm cells)** | R | 0.42271 | 0.48256[a] | 0.49025[a] | 0.63207[a] | 0.45328 | -0.48421[a] | 0.42295 |
| | p | 0.09 | 0.05 | 0.05 | 0.01 | 0.06 | 0.05 | 0.09 |
| **Morphology (% normal spermatozoa, strict criteria)** | R | 0.20193 | -0.15257 | -0.19314 | -0.02607 | 0.58301[a] | -0.35997 | 0.10604 |
| | p | 0.45 | 0.57 | 0.47 | 0.93 | 0.02 | 0.17 | 0.70 |
| **$CMA_3$ positve cells (%)** | R | -0.37677 | -0.41322 | -0.44516 | -0.30598 | -0.48181 | 0.88228[b] | -0.59756[a] |
| | p | 0.14 | 0.10 | 0.07 | 0.25 | 0.05 | <0.0001 | 0.01 |
| **Sperm head morphology at time of $CMA_3$ (% normal head morphology)** | R | 0.70954[a] | 0.64355[a] | 0.65458[a] | 0.45259 | 0.46121 | -0.46693 | 0.76309[a] |
| | p | 0.002 | 0.01 | 0.01 | 0.09 | 0.07 | 0.07 | 0.001 |

Sperm head morphology was compared between the IVF/ICSI group at the two time points. At time point 1 of all the spermatozoa counted, 4.4% have normal, 0.6% macrocephalic and 95.0% amorphous head morphology. At time point 2, 3.9% have normal head morphology, 0.6% are macrocephalic and 99.6% have amorphous head morphology. No significant differences can be detected for the number of spermatozoa with normal head morphology, macrocephalic and amorphous spermatozoa between the IVF/ICSI subgroups (normal: p=0.66; macrocephalic: p=0.89; amorphous: p=0.63) (table 3, figure 6C and D).

After evaluation of the $CMA_3$ staining, sperm head morphology was assessed for the $CMA_3$ positive sperm cells. In the IVF/ICSI group 5-7 days before OPU the $CMA_3$ positive sperm cells show 2.3% spermatozoa with normal head morphology, 1.4% macrocephalic sperm cells and 96.4% amorphous head morphology. For IVF/ICSI on the day of OPU this is 2.1%, 1.6% and 96.3% respectively, again comparable outcomes for both time points. No significant differences can be detected for the distribution of the types of

sperm heads that are fluorescent between the two IVF/ICSI subgroups (normal: p=0.82; macrocephalic: p=0.53; amorphous: p=0.92) (table 4, figure 7C and D).

The number of normal, macrocephalic and amorphous spermatozoa with bright fluorescence were determined and compared between the IVF/ICSI group at the two time points. Of the spermatozoa with normal, macrocephalic and amorphous head morphology 23.3%, 92.0% and 43.1%, respectively, are fluorescent on time point 1. Whereas, 20.2% normal, 95.1% macrocephalic and 41.5% amorphous spermatozoa, respectively, display fluorescence on time point 2. No significant differences can be detected for the number of fluorescent normal, macrocephalic and amorphous sperm heads between the IVF/ICSI subgroups (normal: p=0.68; macrocephalic: p=0.55; amorphous: p=0.82) (table 5, figure 8 C and D).

### 3.4 $CMA_3$ and conventional sperm parameters

Pearson's correlation analysis was performed for all conventional parameters as well as on the $CMA_3$ data for the overall patient population (n=40). Table 7 lists the results of this analysis for the overall study population. Concentration correlates positively with morphology according to strict criteria (R=0.51229, p=0.0007). Good motility is correlated positively with total motility (R=0.97903, p=<0.0001) and viability (R=0.52387, p=0.0006). Total motility has a positive correlation with viability (R=0.47312, p=0.003) and a negative correlation with the number of $CMA_3$ positive cells (R=-0.34307, p=0.03).

**Table 7: Comparison of semen parameters in the overall study population.** Pearson's correlation coefficients are listed as R-values and level of significance as p-values. Correlation is significant at [a] 0.05 level [b] 0.001 level

| | | *Concentration* | *Good motile* | *Total motile* | *Viability* | *Morphology (strict criteria)* | *$CMA_3$ positive spermatozoa* | *Sperm head morphology at time of $CMA_3$* |
|---|---|---|---|---|---|---|---|---|
| **Concentration ($10^6$ spermatozoa/ml)** | R | - | 0.24259 | 0.19134 | 0.09078 | 0.51229[a] | -0.29197 | 0.20274 |
| | p | | 0.13 | 0.24 | 0.58 | 0.0007 | 0.07 | 0.26 |
| **Good motile cells (% grade A+B)** | R | 0.24259 | - | 0.97903[b] | 0.52387[a] | 0.20386 | -0.29234 | 0.15781 |
| | p | 0.13 | | <0.0001 | 0.0006 | 0.21 | 0.07 | 0.38 |
| **Total motile (% grade A+B+C)** | R | 0.19134 | 0.97903[b] | - | 0.47312[a] | 0.18024 | -0.34307[a] | 0.17233 |
| | p | 0.24 | <0.0001 | | 0.003 | 0.27 | 0.03 | 0.34 |
| **Viability (% viable sperm cells)** | R | 0.09078 | 0.52387[a] | 0.47312[a] | - | 0.30449 | -0.15108 | 0.22548 |
| | p | 0.58 | 0.0006 | 0.003 | | 0.06 | 0.36 | 0.21 |
| **Morphology (% normal spermatozoa, strict criteria)** | R | 0.51229[a] | 0.20386 | 0.18024 | 0.30449 | - | -0.29095 | 0.28582 |
| | p | 0.0007 | 0.21 | 0.27 | 0.06 | | 0.07 | 0.11 |
| **$CMA_3$ positive spermatozoa (%)** | R | -0.29197 | -0.29234 | -0.34307[a] | -0.15108 | -0.29095 | - | -0.26852 |
| | p | 0.07 | 0.07 | 0.03 | 0.36 | 0.07 | | 0.13 |
| **Sperm head morphology at time of $CMA_3$ (% normal head morphology)** | R | 0.20274 | 0.15781 | 0.17233 | 0.22548 | 0.28582 | -0.26852 | - |
| | p | 0.26 | 0.38 | 0.34 | 0.21 | 0.11 | 0.13 | |

In table 8 the results of correlation analysis for the IUI group are registered. Good motility is correlated positively with total motility (R=0.97938, p=<0.0001) and viability (R=0.54031, p=0.01). Total motility is also correlated positively to viability (R=0.50342, p=0.02). A significant positive correlation is demonstrated between concentration and morphology according to strict criteria (R=0.51877, p=0.01). No correlation is found between conventional parameters and $CMA_3$.

**Table 8: Comparison of semen parameters in the IUI group.** Pearson's correlation coefficients are listed as R-values and level of significance as p-values. Correlation is significant at [a] 0.05 level [b] 0.001 level

| | | *Concentration* | *Good motile* | *Total motile* | *Viability* | *Morphology (strict criteria)* | *$CMA_3$ positive spermatozoa* | *Sperm head morphology at time of $CMA_3$* |
|---|---|---|---|---|---|---|---|---|
| **Concentration ($10^6$ spermatozoa/ml)** | R | - | 0.01284 | -0.05048 | -0.09984 | 0.51877[a] | -0.20290 | 0.11728 |
| | p | | 0.96 | 0.82 | 0.66 | 0.01 | 0.37 | 0.67 |
| **Good motile cells (% grade A+B)** | R | 0.01284 | - | 0.97938[b] | 0.54031[a] | 0.09451 | -0.34959 | -0.24427 |
| | p | 0.95 | | <0.0001 | 0.01 | 0.68 | 0.11 | 0.36 |
| **Total motile (% grade A+B+C)** | R | -0.05048 | 0.97938[b] | - | 0.50342[a] | 0.05456 | -0.37772 | -0.26173 |
| | p | 0.82 | <0.0001 | | 0.02 | 0.81 | 0.08 | 0.33 |
| **Viability (% viable sperm cells)** | R | -0.09984 | 0.54031[a] | 0.50342[a] | - | 0.29637 | -0.18653 | -0.01067 |
| | p | 0.66 | 0.01 | 0.02 | | 0.18 | 0.41 | 0.97 |
| **Morphology (% normal spermatozoa, strict criteria)** | R | 0.51877[a] | 0.09451 | 0.05456 | 0.29637 | - | -0.10823 | 0.20360 |
| | p | 0.01 | 0.68 | 0.81 | 0.18 | | 0.63 | 0.45 |
| **$CMA_3$ positive spermatozoa (%)** | R | -0.20290 | -0.34959 | -0.37772 | -0.18653 | -0.10823 | - | 0.04339 |
| | p | 0.37 | 0.11 | 0.08 | 0.41 | 0.63 | | 0.87 |
| **Sperm head morphology at time of $CMA_3$ (% normal head morphology)** | R | 0.11728 | -0.24427 | -0.26173 | -0.01067 | 0.20360 | 0.04339 | - |
| | p | 0.67 | 0.36 | 0.33 | 0.97 | 0.45 | 0.87 | |

In table 9 Pearson's correlation coefficients for the IVF/ICSI data 5-7 days before OPU are summarized. Good motility significantly correlates positively with total motility (R=0.98369, p=<0.0001) and viability (R=0.65227, p=0.005), as in the IUI group. Total motility is demonstrated to correlate positively with viability (R=0.65849, p=0.004). Again no correlation could be found between conventional parameters and $CMA_3$. However, $CMA_3$ positivity is negatively correlated with sperm head morphology at time of $CMA_3$ staining (R=-0.58202, p=0.02). Sperm head morphology is also positively correlated with concentration (R=0.60444, p=0.01), good motility (R=0.51751, p=0.04), total motility (R=0.55736, p=0.02) and viability (R=0.51930, p=0.03).

**Table 9: Comparison of semen parameters in the IVF/ICSI group 5-7 days before OPU.** Pearson's correlation coefficients are listed as R-values and level of significance as p-values. Correlation is significant at [a] 0.05 level [b] 0.001 level

| | | *Concentration* | *Good motile* | *Total motile* | *Viability* | *Morphology (strict criteria)* | *$CMA_3$ positive spermatozoa* | *Sperm head morphology at time of $CMA_3$* |
|---|---|---|---|---|---|---|---|---|
| **Concentration ($10^6$ spermatozoa/ml)** | R | - | 0.35397 | 0.33687 | 0.47134 | 0.20426 | -0.32711 | 0.60444[a] |
| | p | | 0.16 | 0.19 | 0.06 | 0.45 | 0.20 | 0.01 |
| **Good motile cells (% grade A+B)** | R | 0.34397 | - | 0.98369[b] | 0.65227[a] | 0.01379 | -0.34800 | 0.51751[a] |
| | p | 0.16 | | <0.0001 | 0.005 | 0.96 | 0.17 | 0.04 |
| **Total motile (% grade A+B+C)** | R | 0.33687 | 0.98369[b] | - | 0.65849[a] | -0.07874 | -0.37793 | 0.55736[a] |
| | p | 0.19 | <0.0001 | | 0.004 | 0.77 | 0.13 | 0.02 |
| **Viability (% viable sperm cells)** | R | 0.47134 | 0.65227[a] | 0.65849[a] | - | 0.13231 | -0.47026 | 0.54930[a] |
| | p | 0.06 | 0.005 | 0.004 | | 0.63 | 0.06 | 0.03 |
| **Morphology (% normal spermatozoa, strict criteria)** | R | 0.20426 | 0.01379 | -0.07874 | 0.13231 | - | -0.42348 | 0.20274 |
| | p | 0.45 | 0.96 | 0.77 | 0.63 | | 0.10 | 0.47 |
| **$CMA_3$ positive spermatozoa (%)** | R | -0.32711 | -0.34800 | -0.37793 | -0.47026 | -0.42348 | - | -0.58202[a] |
| | p | 0.20 | 0.17 | 0.13 | 0.06 | 0.10 | | 0.02 |
| **Sperm head morphology at time of $CMA_3$ (% normal head morphology)** | R | 0.60444[a] | 0.51751[a] | 0.55736[a] | 0.54930[a] | 0.20274 | -0.58202[a] | - |
| | p | 0.01 | 0.04 | 0.02 | 0.03 | 0.47 | 0.02 | |

Table 10 lists the Pearson's correlation coefficients for the IVF/ICSI group on the day of OPU. Good motility demonstrates a significant positive correlation to total motility (R=0.97746, p=<0.0001) and to viability (R=0.49110, p=0.05). Concentration and morphology display a significant positive correlation (R=0.47162, p=0.05). The number of $CMA_3$ positive cells have a significant negative correlation with morphology (strict criteria) (R=-0.48807, p=0.04) and morphology at time of $CMA_3$ evaluation (R=-0.54163, p= 0.03).

**Table 10: Comparison of semen parameters in the IVF/ICSI group on the day of OPU.** Pearson's correlation coefficients are listed as R-values and level of significance as p-values. Correlation is significant at [a] 0.05 level [b] 0.001 level

| | | *Concentration* | *Good motile* | *Total motile* | *Viability* | *Morphology (strict criteria)* | *$CMA_3$ positive spermatozoa* | *Sperm head morphology at time of $CMA_3$* |
|---|---|---|---|---|---|---|---|---|
| **Concentration ($10^6$ spermatozoa/ml)** | R | - | 0.39249 | 0.35538 | 0.25809 | 0.47162[a] | -0.37721 | 0.16879 |
| | p | | 0.11 | 0.15 | 0.32 | 0.05 | 0.12 | 0.52 |
| **Good motile cells (% grade A+B)** | R | 0.39249 | - | 0.97746[b] | 0.49110[a] | 0.26275 | -0.23069 | 0.46249 |
| | p | 0.11 | | <0.0001 | 0.05 | 0.29 | 0.36 | 0.06 |
| **Total motile (% grade A+B+C)** | R | 0.35538 | 0.97746[b] | - | 0.42296 | 0.25816 | -0.30386 | 0.52002[a] |
| | p | 0.15 | <0.0001 | | 0.09 | 0.30 | 0.22 | 0.03 |
| **Viability (% viable sperm cells)** | R | 0.25809 | 0.49110[a] | 0.42296 | - | 0.27606 | -0.09933 | 0.37076 |
| | p | 0.32 | 0.05 | 0.09 | | 0.28 | 0.70 | 0.16 |
| **Morphology (% normal spermatozoa, strict criteria)** | R | 0.47162[a] | 0.26275 | 0.25816 | 0.27606 | - | -0.48807[a] | 0.29062 |
| | p | 0.05 | 0.29 | 0.30 | 0.28 | | 0.04 | 0.26 |
| **$CMA_3$ positive spermatozoa (%)** | R | -0.37721 | -0.23069 | -0.30386 | -0.09933 | -0.48807[a] | - | -0.54163[a] |
| | p | 0.12 | 0.36 | 0.22 | 0.70 | 0.04 | | 0.03 |
| **Sperm head morphology at time of $CMA_3$ (% normal head morphology)** | R | 0.16879 | 0.46249 | 0.52002[a] | 0.37076 | 0.29062 | -0.54163 | - |
| | p | 0.52 | 0.06 | 0.03 | 0.16 | 0.26 | 0.03 | |

### 3.5 $CMA_3$ and outcome in ART

In this study population, fertilization rate was determined, embryo quality was verified and pregnancy rates were calculated. However, due to the limited number of patients enrolled in this prospective cohort study during the 4 month period, these data have not been analyzed statistically, since no valid conclusions can be made.

Demographic data on treatment outcome are listed in table 11. In the overall patient group 56% of oocytes were fertilized (67/120) and 51 top quality embryos developed (42.5%). Six out of 40 patients obtained a pregnancy (pregnancy rate of 15%). The clinical pregnancy rate noted, is also 6/40. For the IUI group a pregnancy rate of 9% is noted (2/22) and a clinical pregnancy rate of 2/22. In the IVF/ICSI group the fertilization rate and the amount of top quality embryos is identical to the overall group. A pregnancy rate of 22% is reached (4/18). No miscarriages were observed resulting in a clinical pregnancy rate of 4/18.

**Table 11: Demographic data on outcome in ART.**

| | ***Overall*** | ***IUI*** | ***IVF/ICSI*** |
|---|---|---|---|
| **Number of patients** | 40 | 22 | 18 |
| **Number of oocytes** | 120 | | 120 |
| **Fertilization rate (% fertilization)** | 67/120 (56) | | 67/120 (56) |
| **Top quality embryos** | 51/120 | | 54/120 |
| **Number of pregnancies** | 6 | 2 | 4 |
| **Pregnancy rate (% pregnant)** | 6/40 (15) | 2/22 (9%) | 4/18 (22%) |
| **Clinical pregnancy rate (% clinically pregnant)** | 6/40 (15) | 2/22 (9%) | 4/18 (22%) |

### 3.6 Summary of the results

In table 12 the results are summarized to give an overview of the most important findings of this study.

**Table 12: Summary of the most important results.** In the IVF/ICSI group T1 is 5-7 days before and T2 is the day of OPU.

| ***Study*** | ***Observation made for:*** | ***Result*** |
|---|---|---|
| **Part 1** | All spermatozoa counted | 39.2% $CMA_3$ positive<br>94.6% with amorphous head |
| | $CMA_3$ positive cells | 95.2% with amorphous head<br>1.8% with giant head<br>3.0% with normal head |
| | Giant sperm heads<br>Amorphous sperm heads<br>Normal sperm heads | 92.7% fluorescent<br>39.8% fluorescent<br>17.0% fluorescent |
| **Part 2** | IUI, IVF and ICSI patients | Same distribution in fluorescence and sperm head morphology |
| **Part 3** | IVF/ICSI T1 versus T2 | All parameters are comparable on T1 and T2 |
| **Part 4** | Overall study population | Negative correlation: $CMA_3$ positivity with total motility |
| | IUI group | No correlations |
| | IVF/ICSI T1 | Negative correlation: $CMA_3$ positivity with sperm head morphology |
| | IVF/ICSI T2 | Negative correlation: $CMA_3$ positivity with sperm head morphology and overall morphology (strict criteria) |

# 4 Discussion

Replacement of histones by protamines during spermiogenesis plays an important role in the packaging process of the nuclear DNA of spermatozoa. Protamination of the chromatin in spermatozoa leads to a high degree in condensation of up to six times that of a mitotic chromosome [37], facilitates sperm motility and protects genetic material from damage [16, 35]. On the other hand, anomalies in chromatin packaging can lead to failure of the sperm cell DNA to decondense after IVF and ICSI and eventually in fertilization failure [2, 40]. During this research project chromatin protamination was evaluated by $CMA_3$ staining. The $CMA_3$ fluorochrome competes with protamines to bind to DNA and hence fluorescence gives an indirect indication of poor chromatin packaging quality [40]. Moreover, the test is straightforward to perform and fluorescence outcome can be evaluated in an all or nothing approach. High $CMA_3$ positivity has been found in spermatozoa of infertile patients [42] and previous studies by other authors indicated a negative correlation between $CMA_3$ values and IVF results [18, 41, 46]. However, these results were not always confirmed by other studies [2, 48].

This study was performed on 40 patients attending the fertility centre for IUI, IVF or ICSI treatment. The focus was on the contribution that $CMA_3$ staining could make to the routine WHO semen analysis of patients entering the ART programme. Our preliminary results that were obtained so far will be discussed.

In a first part of the study evaluation of $CMA_3$ positivity was performed simultaneously with sperm head morphology scoring. Overall, 39.2% of the spermatozoa analyzed by $CMA_3$ staining showed to have poor protamination. Bianchi et al. [2] revealed a higher rate of fluorescence in 55 patient undergoing IVF or subzonal insemination, however their study population included patients with severe oligozoospermia (68.0% fluorescence) and asthenozoospermia (71.5% fluorescence). Franken et al. [42] studied semen form a group of fertile donors as well as from 58 men visiting the andrology laboratory. Fertile donors had a mean $CMA_3$ positivity of 35.8% and for the patients a mean $CMA_3$ positivity was noted ranging from 29.7% to 49.0%. In the latter study $CMA_3$ positivity was negatively correlated with sperm morphology (strict criteria). In the present study, the majority of spermatozoa that fluoresced had an abnormal head morphology (97%), i.e. 95.2% amorphous head structure and 1.8% macrocephalic head structure. Of all the spermatozoa that fluoresced only 3% had a normal head morphology. When focusing on sperm head morphology a first category consisted of spermatozoa with amorphous sperm head morphology having deformities ranging from minimal abnormalities of the

head to completely irregular shape. 39.8% of amorphous spermatozoa demonstrated fluorescence, significantly less than in macrocephalic spermatozoa (92.7%). These results are confirming the work of Iranpour et al. [41] who stated that high $CMA_3$ positivity was observed especially in samples containing high numbers of amorphous spermatozoa. Interestingly, Lee et al. [52] found amorphous sperm cells to have a higher rate of chromosomal abnormalities (26.1%) compared to normal spermatozoa (6.9%). The second category are spermatozoa with macrocephalic sperm head morphology which have an abnormally large head. This subclass of spermatozoa had the highest fluorescence score in all treatment groups: over 90% of the spermatozoa with macrocephalic sperm head morphology fluoresced. These observations are in accordance with the results of Iranpour et al. [41] studying a population of 139 IVF patients and Bianchi et al. [2] who found a high frequency (>75%) of $CMA_3$ positive nuclei among the macrocephalic spermaotozoa in a population of 55 IVF and subzonal insemination patients. Moreover, Yurov et al. [53] observed that the majority of large headed spermatozoa are chromosomally diploid. Possible chromatin decondensation resulting from this lack of protamines in a macrocephalic sperm head could give rise to the large head shape [2]. The third category in the sperm head scoring are the spermatozoa with a normal head shape. Sperm heads are considered normal when they display a smooth, oval shape. Sperm cells with normal head morphology displayed the lowest amount of fluorescence scoring (17.0%). The observations indicate that DNA protamination status is reflected in head morphology of spermatozoa and that some normally shaped spermatozoa can carry defective protamination of DNA.

In a second part of the study, $CMA_3$ positivity was studied in IUI and IVF/ICSI patients: all patients demonstrated the same distribution of fluorescence over the three types of sperm head morphology. One would expect that the IUI group would demonstrate a different distribution, in fluorescence and especially sperm head morphology patterns, than the IVF/ICSI group, since patients are assigned to the different treatments based on morphology (according to strict criteria) and IMC. However, the results indicated that these patients also produce spermatozoa with poor protamination.

Our studies and those of other authors confirm the importance of sperm preparation and selection of morphologically normal spermatozoa before IUI, IVF and ISCI [23, 30-33]. A study by Sakkas et al. [54] demonstrated that when density gradient centrifugation was carried out to isolate motile and morphologically normal spermatozoa, a significant decrease in spermatozoa with both $CMA_3$ positivity and DNA fragmentation could be observed. In IUI and IVF the normal forms should be concentrated and in ICSI a normal

sperm cell should be selected under high microscopic magnification before injection into the oocyte. However, as mentioned before normally shaped spermatozoa may also have poor protamination.

In a third part concentration, motility, viability, morphology (strict criteria), $CMA_3$ positivity and sperm head morphology at time of $CMA_3$ evaluation were compared at two different time points in the IVF/ICSI group, more specifically 5-7 days before oocyte pick-up and at the day of oocyte pick-up. Significant correlations could be found for all parameters at time point one with the respective parameters at time point two. For the IVF/ICSI group no differences could be found between the distribution of sperm head morphology and $CMA_3$ fluorescence patterns at the two days of sperm collection. Accordingly, all patients in these two groups had similar values for sperm head morphology and fluorescence. This could indicate that the sperm analysis obtained, could be typical for each patient. However, since there are only 5-7 days between the two days of sample collection, the two samples produced could be produced in the same spermatogenic wave [55].

In a fourth part the overall patient population, as well as the subgroups, were evaluated for possible relationships between the WHO sperm parameters, $CMA_3$ positivity and sperm head morphology at time of $CMA_3$ evaluation. Any of the WHO sperm parameters is correlated with at least one other WHO sperm parameter in the overall group. $CMA_3$ positivity is negatively correlated only to total motility for the overall population, but no correlation could be found with morphology. Negative correlations between DNA protamination status evaluated by $CMA_3$ staining and motility were previously detected by other studies [18, 41]. However, the finding that there is no correlation between $CMA_3$ positivity and morphology according to strict criteria is contradictory to other studies, because different groups detected a negative correlation between normal morphology and $CMA_3$ positivity [7, 18, 41, 42, 46]. In a previous study carried out on an IVF/ICSI population by Nijs et al. [48] a negative correlation was found between $CMA_3$ positivity and concentration. This result could not be confirmed by the current research project. Carrell et al. [56] formulated two hypothesis about relationships between semen parameters and abnormal protamination: 1) protamine expression may act as a 'checkpoint' during spermiogenesis and abnormal expression of protamines can lead to increased levels of apoptosis which results in a diminished semen quality; 2) abnormal protamine expression can be the result of defective regulators of transcription or translation that also affect genes involved in spermatogenesis [56].

Observations that were made for the overall group could be made for the different subgroups as well, with the exception that no correlations were found with morphology according to strict criteria in the IVF/ICSI group 5-7 days before OPU. However, it was noted that the semen samples used in the IVF/ICSI group carry more abnormalities compared to the IUI group, since there are more correlations between the different WHO parameters including $CMA_3$ positivity. Moreover, for the IUI group no correlations could be found between any of the WHO sperm parameters and poor DNA protamination. An explanation for the difference between the observations in the different subgroups would be that patients which undergo IUI treatment, in general, have better WHO semen parameters, IUI being the first line infertility treatment (figure 1).

In the IVF/ICSI group different observations were made between the group 5-7 days before OPU and the group at the day of OPU. No correlations were found between any of the WHO parameters and $CMA_3$ positivity 5-7 days before OPU. $CMA_3$ positivity on this time point was only negatively correlated to sperm head morphology. However, on the day of OPU significant negative correlations of $CMA_3$ were found with morphology according to strict criteria and with morphology of the sperm head at time of $CMA_3$ evaluation. The different correlations found between the two time points in the IVF/ICSI group can perhaps be explained by a less strict compliance to the abstinence period 5-7 days before the OPU. The abstinence period that should be respected is 2 days. Men might not be complying as strictly to the period of abstinence at this time point, because the sample which is produced on this day is not used for insemination of the oocytes. Evidence has been provided by De Jonge et al. [57] that a short abstinence interval has a negative influence on chromatin packaging, because the percentage of sperm observed with immature chromatin was significantly higher after 1 day's abstinence compared to 5 day's abstinence. Although the WHO values are comparable between the two time points in the IVF/ICSI group, still DNA quality could have been influenced by the short or possibly very long time of abstinence.

Since this study should be considered as a pilot study, it should be taken into consideration that preliminary data are discussed. Correlations and predictive values for $CMA_3$ staining and IUI, IVF and ICSI outcome could not be realized. The study is currently ongoing.

# 5 Conclusion and Synthesis

In summary, spermatozoa with macrocephalic head morphology display fluorescence in 90% of the cells, this score is significantly higher than that of amorphous and normal sperm heads. When looking at the sperm head morphology in general, without taking into account fluorescence patterns, all groups of patients have the same distribution. $CMA_3$ positivity also has the same distribution for the groups studied here. All parameters investigated correlated with the respective parameters for patients evaluated at two different time points and in the overall study population $CMA_3$ positivity only correlates to total motility. However, in the IVF/ICSI group on the day of OPU a correlation could be detected between poor protamination and morphology according to strict criteria as well as sperm head morphology at time of $CMA_3$ staining.

The hypothesis at the start of this research project stated that there would be a correlation between at least one of the conventional parameters and DNA protamination assessed by $CMA_3$ staining. A second part of the hypothesis stated that there would be a correlation between DNA protamination, sperm quality and the fertilization and pregnancy rates of the patients. The first part of this hypothesis is not refuted, since a correlation was found in the overall population between $CMA_3$ positivity and total motility. About the second part of the hypothesis no conclusions can be made due to low patient numbers.

From these results can be concluded that morphologically normal spermatozoa should be selected before IUI and IVF and that a morphologically normal sperm cell should be selected for oocyte injection in ICSI. Another important finding is that all parameters studied seem to be typical for each patient and/or spermatogenic wave.

This research project will continue at the Genk Institute for Fertility Technology to increase patient numbers, in order to reach higher statistical significance and to have the ability to draw solid conclusions on the pregnancy outcome.

Another point of focus in future research should be the period of abstinence. It should be investigated if the differences that were found in the IVF/ICSI group between the two time points are due to a less strict compliance to the two day period of abstinence or if there might be a different reason. So, a good monitoring of the abstinence periods of the patients is necessary when they come to the lab 5-7 days before OPU

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## Supplements

**Supplement 1**

*Human sperm morphological defects (WHO, 1999) [23]*

*Head defects:* - large, small, tapered, pyriform, round

- amorphous
- vacuolated (> 20% of head area)
- small acrosomal area (< 40% of the head area)
- large acrosomal area (> 70% of the head area)
- double heads
- any combination of these

*Neck and midpiece:* - bent neck (> 90%)

- asymmetrical insertion of the midpiece in the head
- thick or irregular midpiece
- abnormally thin midpiece
- any combination of these

*Tail defects:* - short

- multiple tails
- hairpin
- bent tail (> 90%)
- irregular width, coiled
- any combination of these

*Cytoplasmic droplets:*- greater than a third of the head area, usually located on side of the midpiece

**Supplement 2**

McIlvaine buffer, pH 7.0

Solution A: 0.1 mol/l citric acid (19.2 g:q.s. to 1l with distilled water)

Solution B: 0.2 mol/l $Na_2HPO_4$ (28.4 g:q.s. to 1l with distilled water)

pH 7.0: x = 18.2

x ml A + (100 – x) ml B = 100 ml total volume

➔ To make the solution needed to dissolve $CMA_3$ in:

Solution A: citric acid → 0.96 g in 50 ml distilled water

Solution B: $Na_2HPO_4$ → 1.42 g in 50 ml distilled water

9.1 ml A + 40.9 ml B = 50 ml McIlvaine buffer

5 mg $CMA_3$ is dissolved in 20 ml of McIlvaine buffer

The 20 ml of $CMA_3$ solution is divided over 20 tubes of 1 ml and stored in the freezer at -20°C until use.