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EVALUATION OF THE IMPLEMENTATION OF AN ARTIFICIAL INSEMINATION WITH HUSBAND'S SEMEN PROGRAM AT JOAN XXIII UNIVERSITY HOSPITAL OF TARRAGONA

FINAL DEGREE PROJECT

directed by

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The study is done based on external practices carried out at the Joan XXIII University Hospital directed by Dr. Rafael Sánchez Parrilla.

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"Gratitude makes sense of our past, brings peace for today and creates a vision for tomorrow" Melodie Beattie, American writer.

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ABSTRACT

Artificial insemination with husband's semen (*AIH*) is a reproductive technique that involves the deposit of semen, indicted in the laboratory by techniques to improve their quality (swim-up), into the female genital tract carried out with an intrauterine insemination after having conducted a controlled ovarian stimulation. In this study, the effectiveness of the *AIH* program established in the Joan XXIII University Hospital since 2008 was evaluated using a sample of 51 couples. Each of the couples was subjected to a number of insemination cycles; until they achieved pregnancy, until abandonment or until the end of the maximum allowed number of cycles. Anthropological, biochemical and clinical analysis was done with all the couples, mainly based on semen quality pre and post capacitation. Then, a statistical study was performed by correlating different factors that might alter the pregnancy success. Our results show that a smaller amount of triacylglycerol's in women is statistically associated with a greater likelihood of pregnancy ($p=0.035$). There has been a statistical significance between the increased percentage of post capacitation type a spermatozoa associated with an increased likelihood of a positive pregnancy ($p=0.031$). The fact of the decreasing in likelihood of pregnancy when exceed the fourth insemination cycle was corroborated ($p=0.014$). From the obtained results, we can conclude that improving some aspects in males and females using different treatments could improve the effectiveness of the technique and therefore have more chance of reaching our objective, the pregnancy.

1. INTRODUCTION

Currently, there are so many problems related with fertility and some of them are produced by lots of toxic factors that people don't have in their mind[1,2]. Fertility is the ability to reproduce; men who are fertile are able to father children and fertile women are able to get pregnant and carry their baby to full term, with a live birth nine months after conception. This all happens naturally as a result of sexual intercourse.

As we age, our fertility starts to decline[3], particularly the age of the female partner who is trying to conceive. Infertility is therefore much more common in older women[4,5]. Fertility rates initially start to drop as early as the mid twenties, and fall sharply past the age of 35. Under 35, woman have a 92% chance of becoming pregnant within two years of regular, unprotected sex, whereas by 38 years old, chances of becoming pregnant drop to just 77 %. Women trying to get pregnant, without using fertility drugs or *in vitro* fertilization:

- At age 30:
    - 75% will have a conception ending in a live birth within one year.
    - 91% will have a conception ending in a live birth within four years.
- At age 35
    - 66% will have a conception ending in a live birth within one year.
    - 84% will have a conception ending in a live birth within four years.
- At age 40
    - 44% will have a conception ending in a live birth within one year.
    - 64% will have a conception ending in a live birth within four years.

Evidence shows that increased male age is associated with a decline in semen volume, sperm motility, and sperm morphology[6]. In studies that controlled for female age, comparisons between men under 30 and men over 50 found relative decreases in pregnancy rates between 23% and 38% respectively.

Sperm count declines with age, with men aged 50-80 years producing sperm at an average rate of 75% compared with men aged 20-50 years. However, an even larger difference is seen in how many of the seminiferous tubules in the testes contain mature sperm;

- In males 20-39 years old, 90% of the seminiferous tubules contain mature sperm.
- In males 40-69 years old, 50% of the seminiferous tubules contain mature sperm.
- In males 80 years old and older, 10% of the seminiferous tubules contain mature sperm[7,8].

This is thought to be one reason why fertility issues have been increasing steadily over the last 50 years in developed countries; women tend now to leave having a family until later because the pressure to work and have a career is greater. There are a number of factors that can affect fertility in both men and women[9]. Being overweight or obese reduces both male and female fertility. In women, being overweight can affect ovulation, and will not ovulate if they are severely underweight. There are several sexually transmitted infections (SITs) that can cause infertility. For example, *chlamydia* can damage the fallopian tubes in women, and cause swelling and tenderness of the scrotum in men. Another factor is smoking, as well as affecting general and long-term health; smoking can also adversely affect fertility. As exposure to certain pesticides, heavy metals, solvents and some environmental factors can affect fertility, for example in Tarragona, the region of the study, are known to have poorer semen quality than other zones of Spain and it's because the presence of the petrochemical industry near the capital. If either of the couple suffers stress, it may affect coexistence. Stress can contribute to loss of libido (sex drive), which in turn can reduce the frequency of sexual intercourse. Severe stress may also affect female ovulation and limit sperm production. Apart from these factors, others may also affect as alcohol, coffee and drugs[10,11,12].

Around 10% to 15% of couples who want to start a family face problems with their fertility[12]. This may be due to a problem with the male or the female partner, or a combination of both. Although many people spend their early sexual life suppressing their fertility, when they want to have children and cannot, they feel cheated of an important part of normal life and this can cause severe emotional and social issues.

A couple are said to experience infertility when they are having regular, unprotected sex but do not manage to conceive. Some experts and organisations state this issue is true after a year of trying, while others insist that a couple can only be considered infertile after two years of trying.

In some people, physical signs or previous disease point to a clear reason for infertility. A woman who has had pelvic inflammatory disease or endometriosis, and whose fallopian

tubes are blocked, is unlikely to conceive. Similarly, a woman who has gone into menopause prematurely at her early 30s will not be able to get pregnant. In men, undescended testicles can mean that the sperm are not capable of fertilising an ovule because the temperature inside the body is too high for them to mature properly.

In most cases, however, the reasons for infertility are difficult to pin down. Despite many tests, infertility persists when there is no apparent cause. It is also possible for a couple to have one child without any problems and then to experience infertility when they try again a couple of years later. This is defined as secondary infertility, whereas not being able to conceive at all is termed primary infertility[12].

On average, around 85 % of couples under 35 who have regular, unprotected sex will conceive within a year. That percentage rises to 90 % after two years but infertility is more common than you may think. About 25 % of couples go through a period of infertility during a year or longer. Of these, it is estimated that between 10 % and 15 % of couples worldwide have a continuing problem with infertility and may need infertility treatment to start a family. In developed countries, one in six couples seek help after failing to conceive when actively trying to do so.

The first line of advice is usually to keep trying, but after two years most doctors around the world agree that investigations into the possible reasons for infertility should then be carried out. If there is a clearly identifiable medical problem with either the male or female partner, then a diagnosis of infertility can be given fairly quickly, but in many couples the cause of their infertility remains unexplained. Identifiable causes of infertility problems are split evenly between male and female issues, with around 29,1% of problems caused by female partner. 27,1% of problems are caused by the male and a further 25,2% of problems are caused by a combination of issues from both partners, while the rest cannot be attributed to either 15,6%. *(Registre_SEF, 2013)*

1.1. CAUSES OF INFERTILITY

All these factors and the toxic habits can affect the fertility in the male partner, altering the quality of their semen and in the female partner, altering their normal hormone system. It is true that in one sex as in the other can be existing causes related with some pathologies or genetic diseases[9].

1.2. MALE CAUSES

For men, the first step to do is a complete blood analysis and a physical exploration because it's possible that the patient has a hormonal alteration like diabetes mellitus, or can has a testicular problem like orchitis[13,14]. Then, they should have a serology of RPR, HIV, Syphilis, HBV and HCV to discard some SIT's and pathologies. Even, the most important to have a good diagnosis to make an *AIH* a semen analysis is done and here we will focus primarily on this.

Semen is a viscous whitish secretion of the male reproductive organs, containing spermatozoa and consisting of secretions of the testes, seminal vesicles, prostate, and bulbourethral glands. To be fertile the semen must have certain qualities; following ejaculation, semen may thicken to a jelly-like consistency. Within 30 minutes, it typically becomes runny and clear. The thickening and liquefaction are thought to be important for reproduction. It is also important to have a good volume, normal pH, good motility, no agglutination or presence of antibody (Ab), don't present abnormal morphology, have a correct vitality and good concentration or sperm numbers[15]. (*Table 1; p26*)

If some of these qualities fail, some pathology related to semen quality like oligospermia or low relation of sperm/ml of ejaculation may be present, asthenospermia or low motility of sperm, necrospermia or non-viable/dead sperm, teratospermia or sperm with abnormal morphology[16,17]. (*Table 2.1, Table 2.2; p27*)

1.2.1. SEMEN ANALYSIS

All factors studied in the seminal analysis are recorded in a form. (*Annex 1*)

Semen has two major quantifiable attributes:

- The total number of spermatozoa: this reflects sperm production by the testes and the patency of the post-testicular duct system.
- The total fluid volume contributed by the various accessory glands: this reflects the secretory activity of the glands.

The nature of the spermatozoa (their vitality, motility and morphology) and the composition of seminal fluid are also important for sperm function.

One of the things that we are convinced on, it's that is impossible to characterize a man's semen quality from evaluation of a single semen sample[17].

SAMPLE COLLECTION

The sample should be collected after a minimum of 2 days and a maximum of 7 days of sexual abstinence. The man should record the time of semen production and deliver the sample to the laboratory within 1 hour of collection.

Before doing the sample collection, the macroscopic and microscopic examination could be done.

INITIAL MACROSCOPIC EXAMINATION

Liquefaction

Immediately after ejaculation into the collection vessel, semen is typically a semisolid coagulated mass. Within a few minutes at room temperature, the semen usually begins to liquefy (become thinner), at which time a heterogeneous mixture of lumps will be seen in the fluid. As liquefaction continues, the semen becomes more homogeneous and quite watery, and in the final stages only small areas of coagulation remain. The complete sample usually liquefies within 15 minutes at room temperature, although rarely it may take up to 60 minutes or more. If complete liquefaction does not occur within 60 minutes, this should be recorded. We have two parameters to evaluate liquefaction: Normal/Incomplete.

Viscosity

After liquefaction, the viscosity of the sample can be estimated by gently aspirating it into a wide-bore (approximately 1.5 mm diameter) plastic disposable pipette, allowing the semen to drop by gravity and observing the length of any thread. A normal sample leaves the pipette in small discrete drops. If viscosity is abnormal, the drop will form a thread more than 2 cm long. High viscosity can interfere with determination of sperm motility, sperm concentration, detection of antibody-coated spermatozoa and measurement of biochemical markers.

Semen Volume

The volume of the ejaculate is contributed mainly by the seminal vesicles and prostate gland, with a small amount from the bulbourethral glands and epididymides. Precise measurement of volume is essential in any evaluation of semen, because it allows the

total number of spermatozoa and non-sperm cells in the ejaculate to be calculated.

Low semen volume is characteristic of obstruction of the ejaculatory duct or congenital bilateral absence of the vas deferens. High semen volume may reflect active exudation in cases of active inflammation of the accessory organs.

Semen pH

The pH of semen reflects the balance between the pH values of the different accessory gland secretions, mainly the alkaline seminal vesicular secretion and the acidic prostatic secretion. The pH should be measured after liquefaction at a uniform time, preferably after 30 minutes, but in any case within 1 hour of ejaculation since it is influenced by the loss of CO_2 that occurs after production.

If the pH is less than 7.0 in a semen sample with low volume and low sperm numbers, there may be ejaculatory duct obstruction or congenital bilateral absence of the vas deferens a condition in which seminal vesicles are also poorly developed[17].

INITIAL MICROSCOPIC INVESTIGATION

In the initial microscopic investigation, the first step is a microscopic investigation of the aggregation of spermatozoa, agglutination and motility.

Aggregation of spermatozoa

The adherence either of immotile spermatozoa to each other or of motile spermatozoa to mucus strands, non-sperm cells or debris is considered to be nonspecific aggregation (*Figure 1*) and should be recorded as such.

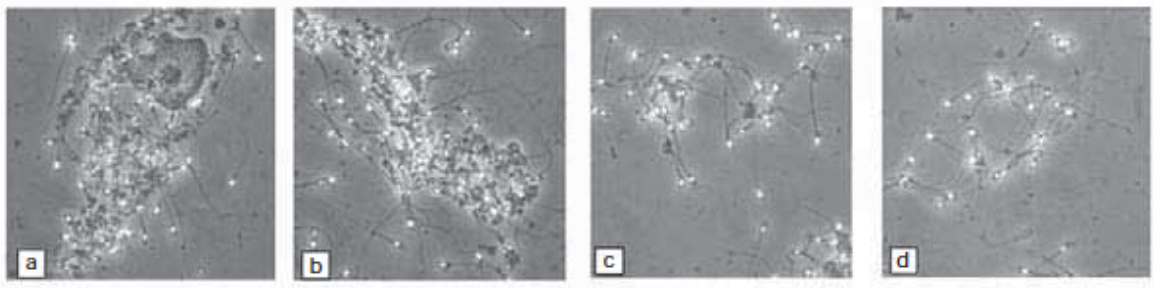

Figure 1. Non-specific aggregation of spermatozoa in semen: Views of spermatozoa aggregated with an epithelial cell (a), debris (b) or spermatozoa (c, d).

Agglutination of spermatozoa

Agglutination specifically refers to motile spermatozoa sticking to each other, head-to-head, tail-to-tail or in a mixed way. The motility is often vigorous with a frantic shaking motion, but sometimes the spermatozoa are so agglutinated that their motion is limited. Any motile spermatozoa that stick to each other by their heads, tails or midpieces should be noted.

The major type of agglutination (reflecting the degree (grades 1–4) and the site of attachment (grades A–E) should be recorded (*Figure 2*):

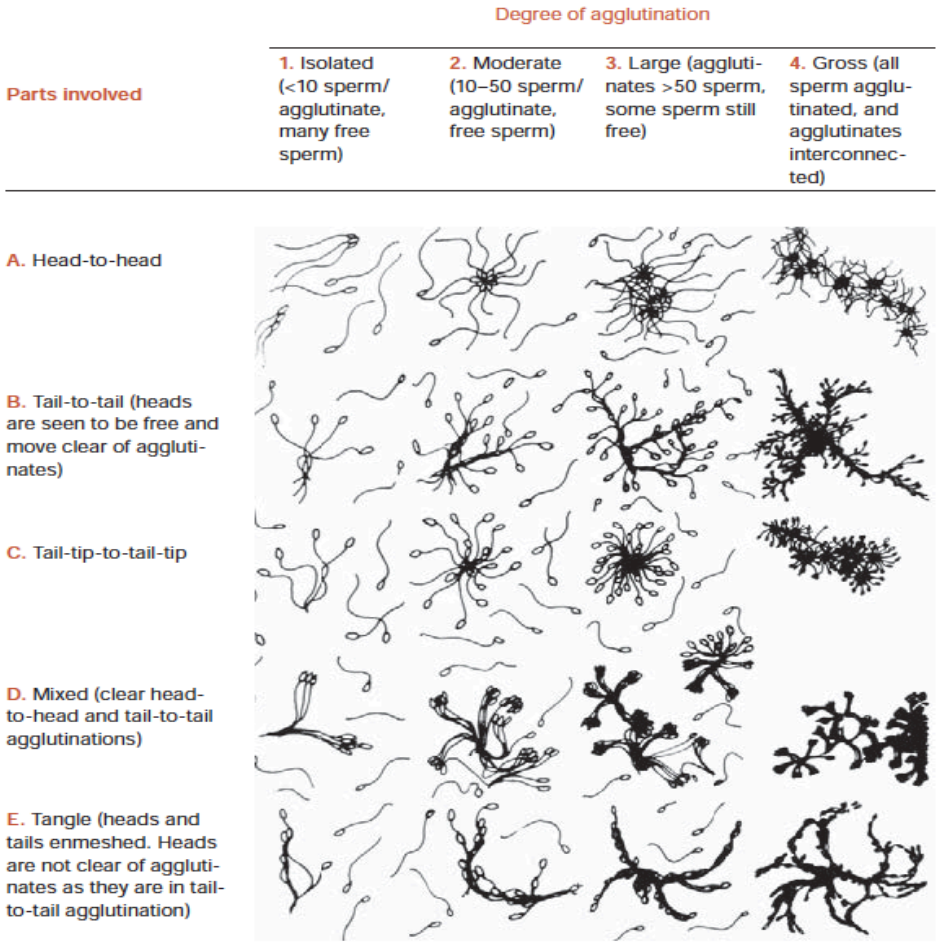

Figure 2. Schematic diagram of different extends of sperm agglutination

The presence of agglutination is not sufficient evidence to deduce an immunological cause of infertility, but is suggestive of the presence of anti-sperm antibodies; further testing is required. Severe agglutination can affect the assessment of sperm motility and concentration.

Motility

A simple system for grading motility is recommended. That distinguishes spermatozoa with progressive or non-progressive motility from those that are immotile. The motility of each spermatozoon is graded as follows:

- Progressive motility (PR or a): spermatozoa moving actively, either linearly or in a large circle, regardless of speed.
- Non-progressive motility (NP or b,c): all other patterns of motility with an absence of progression, swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed.
- Immobility (IM or d): no movement[17].

Vitality

Sperm vitality, as estimated by assessing the membrane integrity of the cells, may be determined routinely on all samples, but is especially important for samples with less than about 40% progressively motile spermatozoa. This test can provide a check on the motility evaluation, since the percentage of dead cells should not exceed (within sampling error) the percentage of immotile spermatozoa. The percentage of viable cells normally exceeds that of motile cells.

The percentage of live spermatozoa is assessed by identifying those with an intact cell membrane, from dye exclusion or by hypotonic swelling. The dye exclusion method is based on the principle that damaged plasma membranes, such as those found in non-vital (dead) cells, and allow entry of membrane-impermeant stains. The hypo-osmotic swelling test presumes that only cells with intact membranes (live cells) will swell in hypotonic solutions.

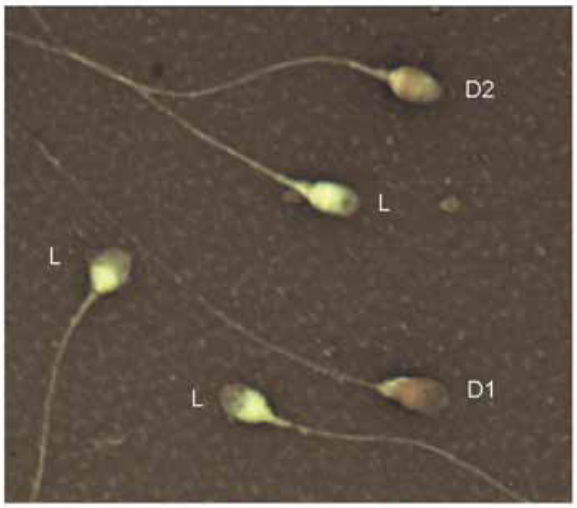

Figure 3. Eosin-Nigrosin smear observed in brightfield optics

We do vitality test using eosin–nigrosin (dye exclusion)[18]. This one-step staining technique uses nigrosin to increase the contrast between the background and the sperm heads, which makes them easier to discern. It also permits slides to be stored for re-evaluation and quality control purposes. In *Figure 3* Spermatozoa with red (D1) or dark pink (D2)

heads are considered dead (membrane-damaged), whereas spermatozoa with white heads (L) or light pink heads are considered alive (membrane intact).

Concentration and Sperm numbers

The terms “total sperm number” and “sperm concentration” are not synonymous. Sperm concentration refers to the number of spermatozoa per unit volume of semen and is a function of the number of spermatozoa emitted and the volume of fluid diluting them. Total sperm number refers to the total number of spermatozoa in the entire ejaculate and is obtained by multiplying the sperm concentration by the semen volume.

The generalization that total sperm number reflects testicular sperm productivity may not hold for electro-ejaculates from men with spinal cord injury, those with androgen deficiency, or for samples collected after prolonged abstinence or partial retrograde ejaculation.

There are two forms of counting sperm numbers: the Neubauer chamber and computer program Sperm Class Analyzer® Casa System (Microptic, Automatic Diagnostic Systems, Barcelona).

Sperm morphology

Human semen samples contain spermatozoa with different kinds of malformations. Defective spermatogenesis and some epididymal pathologies are commonly associated with an increased percentage of spermatozoa with abnormal shapes. The morphological defects are usually mixed. Abnormal spermatozoa generally have a lower fertilizing potential, depending on the types of anomalies, and may also have abnormal DNA. Morphological defects have been associated with increased DNA fragmentation an increased incidence of structural chromosomal aberrations, immature chromatin and aneuploidy. Emphasis is therefore given to the form of the head, although the sperm tail (middle piece and principal piece) is also considered[17,19,20].

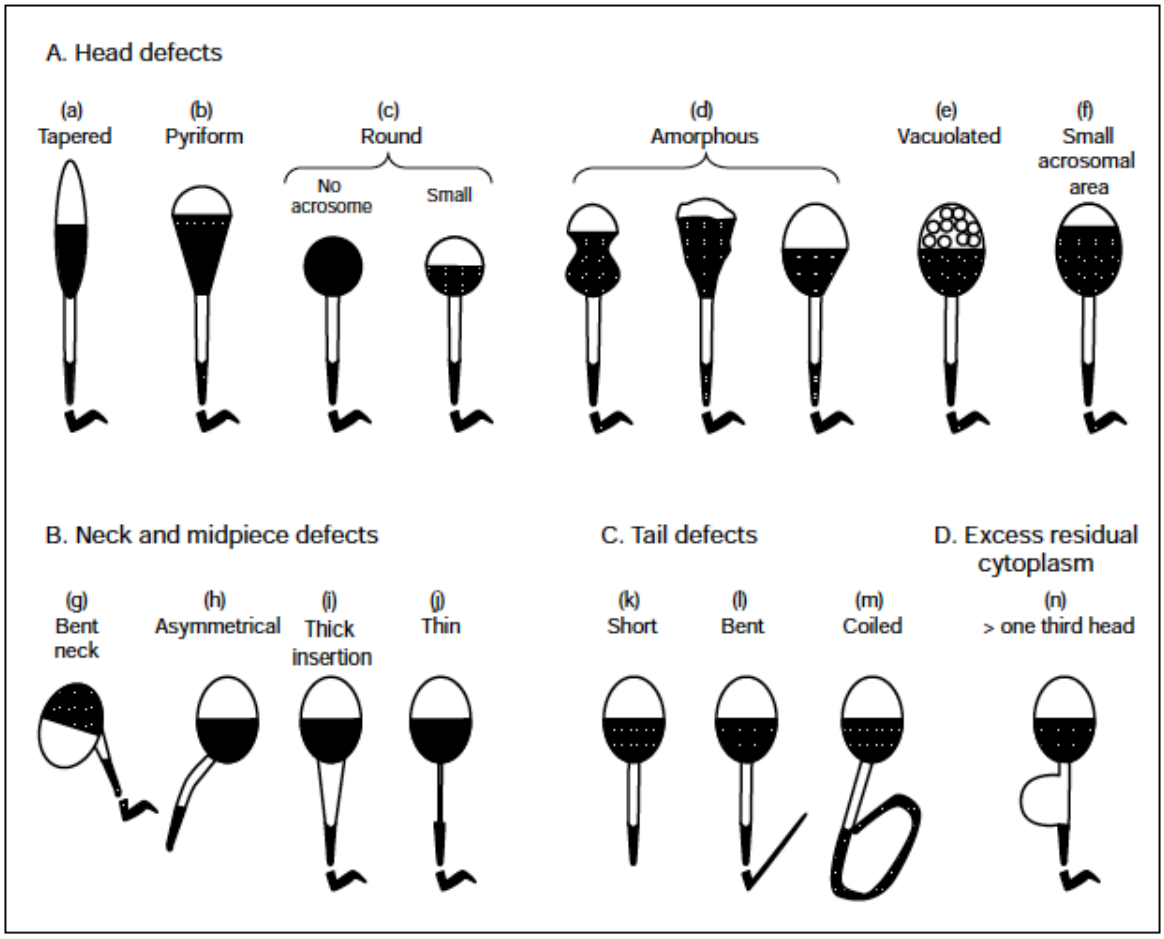

Fig 4. Schematic drawings of some abnormal forms of human spermatozoa

Micrographies of normal and abnormal forms. (*Annex 2*)

1.3. FEMALE CAUSES

For women, the first thing that should be done is a complete blood analysis and a physical exploration. Then, they should have a serology of RPR, HIV, Syphilis, HBV and HCV to discard some SIT's pathologies. Even, the most important to have a good diagnosis to make an *AIH* is performing a hormone analysis on the third and the twenty-first day of the cycle and knows the main disease.

Some of the causes of female infertility are POS, hormonal alteration, ovary alteration, endometriosis, dysmenorrhea, obesity and tubal alteration[2,9,13,14,21].

1.3.1. HORMONES

There are so many hormones involved in regulating female fertility. The following introduces some of the major ones and describes each[12,13,14].

Gonadotropin-Releasing Hormone (GnRH)

The hypothalamus gland in the brain produces this hormone. It is released in short pulses to stimulate the pituitary gland. If the pulses are spaces far apart, the pituitary gland releases follicle-stimulating hormone (FSH). Closely spaced pulses cause the pituitary gland to generate lutenizing hormone (LH).

Follicle-Stimulating Hormone (FSH)

FSH is produced by the pituitary gland. It causes a follicle in the ovary to mature. Each ovarian follicle contains an ovum or ovule. As the follicle matures, it produces hormones to automatically reduce FSH production[22].

Lutenizing Hormone (LH)

Lutenizing hormone is responsible for triggering ovulation. It is also produced by the pituitary gland. A peak in LH levels coincides with ovulation. The now mature follicle releases its ovule[22].

Estrogen

Estrogen is a hormone present throughout the cycle in varying levels. The maturing follicle produces estrogen. This causes the hypothalamus to send signals to the pituitary gland to increase production of lutenizing hormone. Estrogen also stimulates the female reproductive organs to prepare for possible fertilization. This produces observable characteristics that can be monitored to determine fertility[23,24].

Progesterone

Progesterone is produced by the *corpus luteum* after ovulation. The *corpus luteum* is what remains in the ovarian follicle after releasing the ovule. Progesterone signals for the uterus to prepare for a possible implantation by a fertilized ovule. It also sends a signal to the pituitary gland to scale back LH production. The presence of this hormone also causes a subtle elevated shift in core body temperature that can be charted.

Human Chorionic Gonadotropin (HCG)

HCG is produced if the fertilized ovule successfully implants in the uterine wall. It prevents the *corpus luteum* from degenerating so that progesterone and estrogen levels will remain high. HCG is the hormone detected by home pregnancy tests.

Stradiol or E2

Stradiol is the steroid hormone that is produced by the cells lining the ovarian follicles in response to FSH, and the very high levels of stradiol within the leading follicle nourish and mature the ovule. Some stradiol reaches the blood to cause the lining of the uterus to grow, the secretion of ovulatory cervical mucus, and to provide feedback to the brain and pituitary that another cohort of follicles has been recruited and is growing. The level of FSH then falls due to negative feedback by stradiol.

Prolactin

Also known as luteotropic hormone or luteotropin, is a protein secreted from the pituitary gland in response to eating, mating, estrogen treatment, ovulation, and nursing. Prolactin is secreted in a pulsatile fashion in between these events. Prolactin also plays an essential role in: metabolism; regulation of the immune system; and pancreatic development.

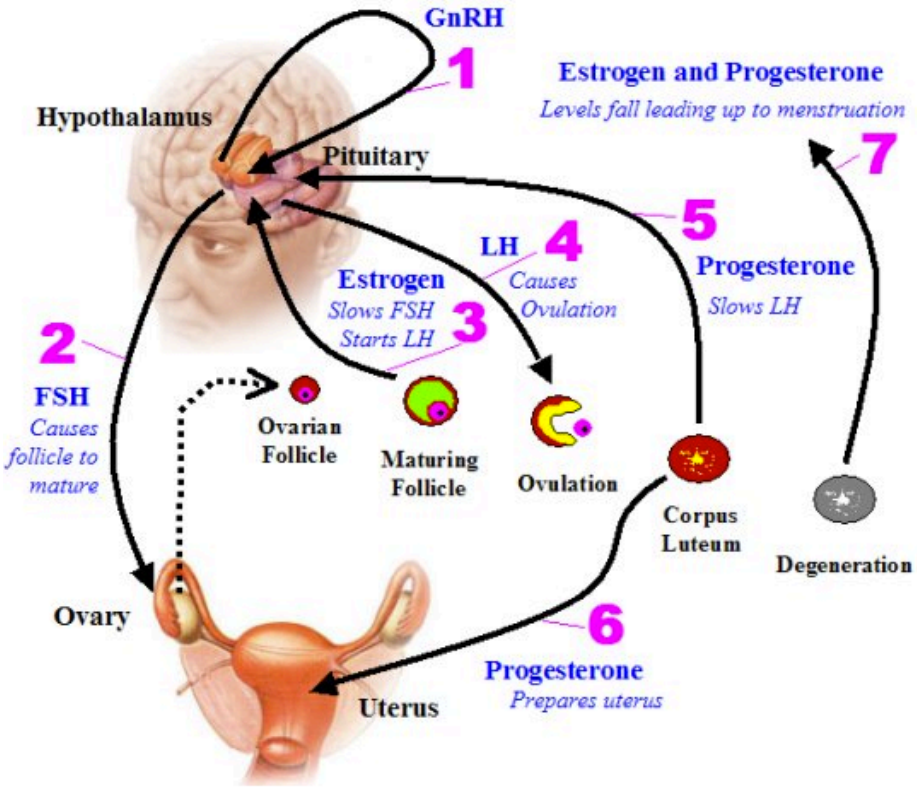

Fig 5. Overview of hormonal events related to female fertility.

Fig 5 it's a schematic representation of hormonal events where:

1. The hypothalamus produces GnRH early in the cycle. This is used to communicate with the pituitary gland and start production of FSH.
2. In response to GnRH, the pituitary gland begins production of FSH. This hormone stimulates the ovaries and causes an ovarian follicle to begin to mature.
3. As the follicle matures, FSH levels decrease. The maturing follicle produces estrogen that feeds back to the hypothalamus. The GnRH production changes frequency and causes the pituitary gland to begin producing lutenizing hormone.
4. A surge in LH levels causes the mature follicle to release its ovule. This event is known as ovulation. The ovule proceeds down the fallopian tube awaiting possible fertilization.
5. The portion of the follicle that remains after ovulation is the *corpus luteum*. It produces progesterone that feeds back to the brain to reduce LH levels.

6. Progesterone also causes the uterus to prepare for possible implantation.
7. Unless pregnancy results and HCG is produced, both progesterone and estrogen levels fall as the corpus luteum degenerates. This causes the body to prepare for menstruation and the start of another cycle if pregnancy does not result.

1.4. PROSPECTS

In the past, infertility had to be accepted as a fact of life but the development of modern infertility treatments has brought about a huge change. Couples experiencing infertility are now able to seek help and advice to find out what is causing their problem, and to have infertility treatments to allow them to have one or more children. This can be an emotionally exhausting process, and it can be costly depending on which country you live in and how easily you can access infertility treatments. However, at least couples facing infertility today have options and the good news is that although up to 15 % of couples will have infertility problems, around 90 % of these problems can be successfully overcome by the range of infertility treatments available today.

One of the options to try to solve these fertility problems is an *AIH* that starts with male sperm capacitation:

Swim-up is the technique used to capacitate male sperm and one of the most commonly used techniques for sperm capacitation. Swim up can be performed using a cell pellet or using a liquefied semen sample. The purpose of this is to isolate the majority of motile sperm ejaculated and rid the sample of most of the dead sperm and debris. The motile sample is then used to inseminate the ovule. On the day of ovule retrieval, have the male partner produce a semen sample in a sterile sample cup. The male partner should be told to abstain from ejaculation for 48-72h before the swim-up.

While it is the swim-up also done during days a hormone stimulation called ovarian controlled stimulation (OCS)[20,27]. OCS is the process of inducing ovarian follicular development and oocyte maturation using medications. The process of ovarian stimulation for AIH involves at least two, and usually three stages:

1) Medications (gonadotrophin or anti-oestrogens) are administered to stimulate the growth of ovarian follicles which then begin the preliminary maturation of oocytes.

2) Medications to prevent surges in lutenising hormone (LH) (the gonadotrophin which regulates oocyte release from follicles) are administered to ensure that oocytes remain within their follicles, rather than being released into the fallopian tubes.

3) Human chorionic gonadotrophin (HCG) is administered to stimulate the final maturation of oocytes, a process that takes just over 36 hours, following which the ovarian follicles rupture and release oocytes.

The first phase of a stimulation cycle coincides with the follicular phase of a normal menstrual cycle and is designed to induce the maturation of numerous ovarian follicles, simultaneously. The majority of ovarian follicles typically die in the first few days of the cycle, as levels of the gonadotrophins follicle stimulating hormone (FSH) and lutenising hormone (LH) decrease. As such, to enable numerous ovarian follicles to continue growing, it is necessary to artificially increase gonadotrophin concentrations. The first phase of a stimulated cycle therefore involves the administration of gonadotrophin-containing compounds or compounds which can stimulate natural gonadotrophin production. The aim is to overcome the decline in FSH and LH levels (and subsequently the death of all but the dominant ovarian follicle), a decline which generally occurs on day 5-7 of the menstrual cycle.

The medications most commonly used to stimulate ovarian follicular growth are:

Clomifene citrate

Clomifene Citrate a non-steroidal estrogen antagonist. Clomifene citrate works by blocking serum stradiol feedback, which induces increased FSH secretion. Clomifene is administered between days 1-5 of the menstrual cycle, in doses of 100-150mg, for five days. The drug regimen is cheap and medications are taken orally[28,29].

Gonadotrophins

Gonadotrophins there are a range of gonadotrophin containing compounds used in ovarian stimulation and they are the principle ovarian stimulation agents used in AIH. Gonadotrophin administration (starting dose from 100-300 IU/day) typically leads to the growth of numerous ovarian follicles and collection of numerous mature oocytes[29,30]. Gonadotrophin administration may commence either in the late luteal phase of the previous menstrual cycle or in the early follicular phase. The appropriate dose will vary from woman to woman. The most commonly used gonadotrophin contain compounds like recombinant FSH[31].

The treatment chose for the AIH is the use of gonadotrophins, but there are so many drugs to stimulate hormones and ovule development.

It is possible to do the AIH when OSC and swim-up are both carried out.

1.4.1. ARTIFICIAL INSEMINATION WITH HUSBAND'S SEMEN

The artificial insemination with husband's semen is the term used for the placement of the husband's sperm in the female reproductive tract, exactly in this type of insemination, in the uterine with an intrauterine insemination (IUI). After the insemination is necessary do the swim-up to capacitate male sperm and the hormone stimulation in female[20]. All data of AIH analysis was recollected on a form. (*Annex 3*).

IUI is a process by which sperm are placed past the cervix and directly inside the uterus. With natural intercourse, sperm are most concentrated in the cervix and only the most motile ones make their way through the uterus to the fallopian tubes. With IUI, a concentrated suspension of sperm is introduced through the cervix directly into the uterine cavity. This allows large numbers of sperm to reach the fallopian tubes, where fertilization can then occur. This procedure requires that the sperm be "washed" first. That is, the sperm must be separated from the semen (seminal fluid can irritate the uterus, causing severe cramping and pain). Sperm washing is performed under strictly sterile conditions and a variety of safeguards are maintained to insure that semen samples cannot be switched.

The AIH is recommended for couples who have been unable to become pregnant for a minimum of 12 months. The number of *AIH* cycles that we can do to the couple depends on various factors but there is evidence in certain studies that the technique can have good results until the sixth cycle[32]. There is other evidence that the rate of gestation decreases significantly from the fourth cycle[20,33,34].

Many of publications studies of factors can positively or negatively affect *IAH* results, agree in indicating as bad prognostic factors: women age (>38 years old), diminished ovarian reserve, presence of tubal factor, endometriosis, sperm quality (REM<6X10^6/mL), infertility duration (>6 years) and the number of treatment cycles (>4).

Good prognostic factors are: woman age (<38 years old), multifollicular ovulation, the controlled ovary stimulation, absence of tubal factor or endometriosis, good sperm quality (>6milion/ml of motility sperm) and the number of treatment cycles (<4)[35,36].

This technique is offered by the Joan XXIII hospital in Tarragona since 2008. We are going to evaluate their effectiveness and the relation of all semen quality, biochemical and anthropological data with negative or positive pregnancy.

2. OBJECTIVES

The objective of the study is to evaluate the effectiveness of the system of artificial insemination with husband's semen implemented Joan XXIII University Hospital in 2008.

The study of how can affect aspects like quality of semen (motility, concentration, sperm numbers, pH or liquefaction) pre and post capacitation, male or female pathologies to the achievement of pregnancy.

To relate positive and negative pregnancy results with different variables as causative of fertility, biochemical parameters and toxic habits.

To compare the *AIH* program with the Spain Fertility Society (SEF).

3. MATERIALS AND METHODS

3.1. ETHICS STATEMENTS AND DATA COLLECTION

All information has been found in different programs of the Joan XXIII hospital. First of all we took the patient data from paper files. Collected and confirmed the data by the program SINERGI® (Sinergi Software, PA) and the hospital intranet. To see the patient's historical information and confirm the diagnostic use the program SAP® (SAP software 13.5, Chicago). All information has been used with complete confidentiality and meeting the standard law "LEY ORGÀNICA 15/1999, DE 13 DE DICIEMBRE, DE PROTECCIÓN DE DATOS DE CARÁCTER PERSONAL[37].

3.2. COUPLES IN THE STUDY AND DATA COLLECTION

The study population consists of 83 couples that have made a total of 298 AIH's from 2008 to 2014. Many of this population are currently underway and therefore cannot be included in the study. Other patients were excluded because of missing data. Most of the samples were analysed previous to the beginning of the study but 25 were analysed during the study.

After the removal of samples of the population, the study sample is in a total of 51 couples that have done a total of 196 AIH.

All the data were collected from patients attending the Laboratory Analysis (ICS Camp of Tarragona) and the gynecology area of Joan XXIII University Hospital of Tarragona.

The 51 couples are divided between men and women. The men group has a characteristic data that are composed from all of these variables:

- Gender.
- Positive/negative pregnant.
- Age.
- Semen analysis composed by: Concentration, Vitality, Motility, Liquefaction, pH, Morphology, Viscosity and Volume.
- All the AIH cycles with their motility, immobility and concentration.
- Hormones: LH, FSH, Testosterone, TSH and T4.
- Biochemical parameters: TAG, Cholesterol and glucose.
- Positive/negative smoking.

- All the diagnostics.

The women group has a characteristic data that are composed from all of these variables:

- Gender.
- Positive/negative pregnant.
- Age.
- Hormones: LH, FSH, Testosterone, TSH, T4, Estradiol, Prolactin and Progesterone.
- Biochemical parameters: TAG, Cholesterol and glucose.
- Positive/negative smoking.
- All the diagnostics.

3.3. MALE STUDY

Health staff makes the blood analytic and the physical exploration. The blood analytic parameters results were obtained by the ADVIA 1800® Clinical Chemistry System (Bayer Diagnostics, Tarrytown, NY) for biochemical parameters and by the ADVIA Centaur® System (Bayer Diagnostics, USA) analyzer for the serological parameters. Data was automatically exported to the database Sinergi® (Sinergi Software, PA), where data was available for download. For physical or pathology diagnosis the results were obtained from SAP® (SAP software 13.5, Chicago) program. Both Sinergi® and SAP® programs were internal-use software's of the HUJXXIII.

3.3.1. SEMEN ANALYSIS

Semen analysis involves the following steps. All procedures of semen analysis are performed on the model of the WHO[17].

Sample should be collected at home or at the clinic (better option) by always between 20ºC and 37ºC, in a container made of glass or plastic that clinic give to you and with trying to have the most sterility conditions.

In the first 5 minutes:

- Placing the specimen container on the bench or in an incubator (37 °C) for liquefaction.

Between 30 and 60 minutes:

- Assessing liquefaction and appearance of the semen.
- Measuring semen volume.
- Measuring semen pH.
- Preparing a wet preparation for assessing microscopic appearance, sperm motility and the dilution required for assessing sperm number.
- Assessing sperm vitality (if the percentage of motile cells is low).
- Making semen smears for assessing sperm morphology.
- Making semen dilutions for assessing sperm concentration.
- Assessing sperm number.
- Preparing spermatozoa for the immunobead test (if required).
- Centrifuging semen (if biochemical markers are to be assayed).

Within 3 hours:

- Sending samples to the microbiology laboratory (if required).

After 4 hours:

- Fixing, staining and assessing smears for sperm morphology.

INITIAL MACROSCOPIC EXAMINATION

Liquefaction

1. Macroscopic observation about the sample to discard the existence of heterogeneity or pollutants particles.

Viscosity

1. After liquefaction, take a wide-bore pipette and aspirate the semen to another tub.
2. Measure the thread dropping the drop the other tub.

Semen Volume

1. Collect the sample directly into a modified graduated glass with a pipette.
2. Read the volume directly from the graduations in mL.

Semen pH

Semen pH is measured with pH testing strips (macherey-nagel™, MN, Stamford).

1. Mix the semen sample well.
2. Spread a drop of semen evenly onto the pH paper.
3. Wait for the colour of the impregnated zone to become uniform (<30 seconds).
4. Compare the colour with the calibration strip to read the pH

INITIAL MICROSCOPIC INVESTIGATION

To make the initial microscopic investigation we need a phase-contrast microscope. To investigate all the factors to do the preparation of the sample to put into the phase-contrast microscope to analyse in the program Sperm Class Analyzer® Casa System (Microptic, Automatic Diagnostic Systems, Barcelona):

1. Connect the Pratcal® (PlatCal v2.1, Microptic, Barcelona) to the computer and put on the phase-contrast microscope.
2. When the Pratcal® reach 37º of temperature put the Countess® Cell Counting Chamber Slides (Invitrogen™, life technologies™, USA) into.
3. Pipetted 0,3 µL into one space of the chamber after moving the sample up and down ten times.
4. Focus the spermatozoa with the 10x magnification and take on the phase contrast to past the image to the computer to SCA.
5. Take a minimum of four camps and the program gives the results for next parameters: Aggregation of spermatozoa, Agglutination of spermatozoa, Motility and Concentration and Sperm numbers.

Vitality

1. Mix the semen sample well.
2. Remove a 50 µl aliquot of semen and mix with an equal volume of eosin-nigrosin suspension, in a porcelain spot plate well, and wait for 30 seconds.
3. Remix the semen sample before removing a replicate aliquot and mixing with eosin-nigrosin and treating as in step 2 above.
4. For each suspension make a smear on a glass slide and allow it to dry in air.
5. Examine immediately after drying, or later after mounting with a permanent non-aqueous mounting medium. Examine each slide with brightfield optics at 1000x

magnification and oil immersion.

6. Tally the number of stained (dead) or unstained (vital) cells with the aid of a laboratory counter.
7. Evaluate 100 spermatozoa in each replicate, in order to achieve an acceptably low sampling error.

Sperm morphology

1. Preparing a smear of semen on a Pre-Stained Morphology Slide (Advanced Meditech International, AMI).
2. Air-drying, fixing and staining the slide.
3. Mounting the slide with a coverslip if the slide is to be kept for a long time.
4. Examining the slide with brightfield optics at x1000 magnification with oil immersion.
5. Assessing approximately 100 spermatozoa per replicate for the percentage of normal forms and abnormal forms.
6. Comparing replicate values to see if they are acceptably close: if so, proceeding with calculations; if not, re-reading the slides.

3.3.2. REFERENCE VALUES AND SEMEN NOMENCLATURE

Men whose semen characteristics fall bellowed the lower limits that we put here are not necessarily infertile.

To do the diagnostic:

1. Compare all the results with the corresponding value in *Table 1*.
2. Do the diagnostic with pathologies in *Table 2.1*, *Table 2.2*.

Table 1. Lower reference limits for semen characteristics

Parameter	Lower reference limit
Semen volume (ml)	1.5 (1.4–1.7)
Total sperm number (106 per ejaculate)	39 (33–46)
Sperm concentration (106 per ml)	15 (12–16)
Total motility (PR + NP, %)	40 (38–42)
Progressive motility (PR, %)	32 (31–34)
Vitality (live spermatozoa, %)	58 (55–63)
Sperm morphology (normal forms, %)	4 (3.0–4.0)
Other consensus threshold values	
pH	>7.2
Peroxidase-positive leukocytes (106 per ml)	<1.0
MAR test (motile spermatozoa with bound particles, %)	<50
Immunobead test (motile spermatozoa with bound beads, %)	<50
Seminal zinc (mol/ejaculate)	> 2.4
Seminal fructose (mol/ejaculate)	> 13
Seminal neutral glucosidase (mU/ejaculate)	> 20

Table 2.1. The possible diagnostics that the male partner of the couple can have. WHO nomenclature for semen variables. Nomenclature related to semen quality.

Medical Name	Description	Limit reference
Aspermia	Ejaculate does not emit any semen.	<1.5 ml
Oligospermia	Low sperm/ml of semen	<15 milion/ml
Azoospermia	Complete absence of semen	<39 millons
Asthenospermia	Low motility of sperm	<32% a+b <40% a+b+c
Necrospermia	Non-viable / Dead sperm	<58% in live
Teratospermia	Sperm with abnormal morphology	<4% of normal sperm

Table 2.2. The possible diagnostics that the male partner can have. WHO nomenclature for semen variables. Nomenclature related to semen quality.

Medical Name	Description
Normospermia	Total number (or concentration) of spermatozoa, and percentages of progressively motile (a+b) and morphologically normal spermatozoa, equal to or above the lower reference limits
Asthenoteratospermia	Percentages of both progressively motile (PR) and morphologically normal spermatozoa below the lower reference limits
Oligoasthenospermia	Total number (or concentration) of spermatozoa, and percentage of progressively motile (a+b) spermatozoa, below the lower reference limits.
Oligoasthenoteratospermia	Total number (or concentration) of spermatozoa, and percentages of both progressively motile (PR) and morphologically normal spermatozoa, below the lower reference limits
Oligoteratospermia	Total number (or concentration) of spermatozoa, and percentage of morphologically normal spermatozoa, below the lower reference limits

3.4. FEMALE STUDY

For women, the first thing you should do is a complete blood analysis and a physical exploration done by the doctor. Then, they should have a serology of RPR, HIV, Syphilis, HBV and HCV and a hormonal analysis. The blood analysis parameters results were obtained by the ADVIA 1800® Clinical Chemistry System (Bayer Diagnostics, Tarrytown, NY)() for biochemical and hormonal parameters and by the ADVIA Centaur® System (Bayer Diagnostics, USA) analyzer for the serological parameters.

3.5. SPERM CAPACITATION: SWIM-UP

We take the following steps to process the sample:

1. Place the cup containing semen at room temperature to liquefy for least 45 min.
2. After 45 min, check to see if sample has liquefied and if it isn't adds the sample to a cup with chymotrypsin solution.
3. Measure the volume of semen with a serological pipet after the sample has liquefied.
4. Place two drops of semen in warmed slides for motility assessment.
5. Add two 25 µL semen sample to test tubes for concentration assessment.
6. Divide the sperm sample into four conical tubes (centrifuge tubes) and adds PureSperm wash (Nidacon, Swedish "Gotheburg") equal to two times the volume of semen to each tube.
7. Vortex the tubes gently and centrifuge twice at 200g, each for 5 min.
8. Once the tubes stop spinning, remove supernatant with a sterile Pasteur pipet and make sure the pellet remains.
9. Slowly add 250 µL of sperm-washing medium to each tube and loosely cap them. Place the rack of tubes in the incubator for 75 min to allow the sperm to "swim-up".
10. After 75 min, remove tubes from the incubator. Remove the supernatant from all tubes with a sterile Pasteur pipet and place in a small sterile snap-cap tube. Determine sperm motility and concentration.
11. Calculate the insemination volume.
12. Place sperm sample back in the incubator until insemination.

3.6. STATISTICAL TREATMENT

The normality of all the variables was analysed using the test of Kolmogorov-Smirnov. Taking together the sample size, and the results obtained in normality test, it was decided to use a non-parametric statistical analysis.

The differences between the mean of distinct groups of study were analysed using the test Mann-Whitney U. At the same time, Rho the Spearman was used for the analysis of bivariate correlations.

Finally, those variables statistically significant found in univariate analysis was studied if its significance was independently associated with pregnancy. We used for that a multivariate binary logistic regression method.

To carry out the statistical studies SPSS program for Windows version 19.0 (SPSS Inc., Chicago, IL, USA) was used.

A p-value of less than 0.05 was considered to be statistically significant. In each table the significant differences were individually specified.

4. RESULTS

CHARACTERISTICS OF THE STUDY POPULATION

First of all, a descriptive analysis of the study population was done to determine general aspects, shown in *Table 8*. The study consists of 196 inseminations spread between 51 couples that have been a total of 28 negatives (54.91%) and 23 positives (45.09%). Within the positive pregnancy have a total of 6 abortions. We can appreciate that women are the main cause of infertility in our study.

Table 8. Detailed information about the study population before the excluded criteria was added.

GENERAL DATA POST ELIMINATION		% per cycle	% per couple
Total insemination	**196**	**n/a**	**n/a**
Total couples	**51**	**n/a**	**n/a**
Total negatives	**28**	**16.66**	**54.91**
Total positives	**23**	**11.73**	**45.09**
Abortion	**6**	**-**	**-**
Final negatives	**34**	**17.34**	**66.66**
Final positives	**17**	**8.67**	**33.33**

In the table the values with and without take into account abortions number have been added. When referring to the value considering abortions, indicated with Final. Final Negatives means: total number of negative+abortions. Final positives means: total number of positives-abortions.

Therefore, *Table 9* summarizes the classification of the different causes and abundance of each. As we can see in *Table 9*, the female is the predominant cause with 22 cases (43.14%) and the most common diagnosis is POS. Also to highlight, the presence of unknown cases was n=7 (11.76%). There are 4 cases in which the male is the unique cause of infertility and 18 where the cause together is both the male and female. It's important that in all the cases the motility was altered, because it will be the main variable to study afterwards.

Table 9. Classification of the main causes of infertility and their subcategories into each group.

	Number of patients	Positive	Negative
CM	**4 (7.8%)**		
Oligoasthenospermia	**1**	**0**	**1**
Asthenospermia	**2**	**2**	**0**
Asthenoteratospermia	**1**	**0**	**1**
CF	**22 (43.1%)**		
Obesity	**1**	**1**	**0**
Hormonal alteration	**5**	**2**	**3**
Tromp alteration	**1**	**1**	**0**
Ovary alteration	**4**	**1**	**1**
POS	**6**	**4**	**2**
Salpingitis	**1**	**0**	**1**
Asherman	**1**	**0**	**1**
A.Hormone + dysmenorrhea	**1**	**1**	**0**
A.Ovary + dysmenorrhea	**2**	**0**	**2**
MIX	**18 (35.3%)**		
Oligospermia + Endometriosis	**1**	**0**	**1**
Asthenospermia + Endometriosis	**1**	**0**	**1**
Oligoasthenospermia + A.ovary	**2**	**1**	**1**
Asthenospermia + POS + obesity	**1**	**1**	**0**
Asthenospermia + A.ovary	**1**	**0**	**1**
Asthenoteratospermia + mioma	**2**	**0**	**2**
Oligoasthenospermia + POS	**1**	**0**	**1**
Asthenospermia + POS	**7**	**4**	**3**
Normospermia + dysmenorrhea	**1**	**0**	**1**
Asthenospermia + dysmenorrhea	**1**	**1**	**0**
UK	**7 (13.8%)**	**2**	**4**

In the table shown the different types of causes that can affect fertility. Abbreviations: CF: cause feminine; CM: cause masculine; Mix: mixed cause; UK: cause unknown. All the causes less unknown show the different diagnostics that it has and the number of patients that have it.

STUDY OF THE EFFECT OF CLINICAL VARIABLES ON PREGNANCY

The first study is based on how clinical factors can impact on pregnancy (positively or negatively). The main factors to consider are female and male age, female and male cholesterol, female and male glucose, female and male triacylglycerol's, shown in *Table 10*. These factors have been studied because it is believed they could affect pregnancy. One of them, age, affects pregnancy in some related studies but in this study it hasn't been statistically significant.

Of all the factors studied the female TAG stands out because it has statistically significant differences between the positive and negative results (p=0.032). TAG's in negative cases up to 140.28 ± 88.39 and in positive cases are reduced to 85.09 ± 34.02.

Table 10. Biochemical and Age characteristics related to pregnancy.

VARIABLE		P (n=23)	N (n=28)	*p*
AGE	**Male**	35.09 ± 4.35	36.61 ± 5.95	0.470
	Female	34.61 ± 4.64	33.75 ± 4.14	0.582
CHOLESTEROL	**Male**	175.93 ± 53.50	193.30 ± 47.67	0.164
	Female	184.44 ± 34.33	188.32 ± 38.64	0.985
GLUCOSE	**Male**	99.87 ± 29.07	92.53 ± 13.96	0.280
	Female	88.35 ± 17.12	90.21 ± 21.27	0.992
TAG	**Male**	132.87 ± 89.56	154.93 ± 94.75	0.389
	Female	85.09 ± 34.02	140.28 ± 88.39	0.032

Comparison of means between positive and negative of the biochemical and anthropological factors. Continuous variables are expressed as mean ± SD. Comparison of mean age ± SD (male and female) is expressed in years; Comparison of mean cholesterol ± SD (male and female) is expressed in mg/dL (same in glucose and TAG). Analysis is determined using a non-parametric Mann-Whitney U test. A p-value less than 0.05 is considered as statistically significant in all analysis. Abbreviations: SD: Standard deviation; P: positive; N: negative.

Therefore, in *Table 11* another important anthropological factor was studied, which has had repercussions in other studies to discuss later. The studied factor was the snuff and it's relation to get a positive or negative pregnancy, assessment in male and female. The results haven't displayed a statistically significant relationship (p>0.05) of snuff with positive or negative pregnancy.

Table 11. Analysis of the influence of snuff in pregnancy results.

FEMALE SMOKER*PREGNANT					
		PREGNANCY		Total	*p*
		NEGATIVE	POSITIVE		
FEMALE SMOKER	NO	16 (31.4%)	16 (31.4%)	32(62.8%)	0.361
	YES	12 (23.5%)	7 (13.7%)	19 (37.2%)	
Total		28 (54.9%)	23 (45.1%)	51	
MALE SMOKER*PREGNANT					
		PREGNANCY		Total	*p*
		NEGATIVE	POSITIVE		
MALE SMOKER	NO	20 (39.2%)	14 (27.4%)	34 (66.6)	0.426
	YES	8 (15.7%)	9 (17.6%)	17 (33.3)	
Total		28 (54.9%)	23 (45.1%)	51	

Percentage of numbers of cases is showed in parenthesis. The total number of cases is n=51 in female and n=51 in male. Analysis is determined using a Chi Square Test and descriptive statistics.

As has been said, it hasn't been statistically significant in any case and also correlated with expected results.

RELATIONSHIP BETWEEN THE CAUSE OF INFERTILITY AND PREGNANCY

To investigate differential results on pregnancy depending on the cause of infertility the different diagnosis were grouped into four groups. Groups in which women had some pathology and men were normal were called Female cause. Groups, in which men had some pathology and women were normal, were called Male cause. Groups, in which both had pathology, were called Mixed cause. Finally, groups in which both had a normal diagnosis were called Unknown. The results indicate that the Female cause is a tendency, but nothing statistically significant exists, as can see in *Table 12*. The low number of couples in each group didn't permit to perform a good analysis.

Table 12. Analysis of the influence infertility causes in pregnancy results.

INFERTILITY CAUSANT*PREGNANT					
		PREGNANCY		Total	*p*
		NEGATIVE	POSITIVE		
CAUSE OF FERTILITY	MIX	11 (21.5%)	7 (13.7%)	18 (35.3%)	0.597
	CM	2 (3.9%)	2 (3.9%)	4 (7.8%)	
	CF	10 (19.6%)	12 (23.5%)	22 (43.1%)	
	UK	5 (9.8%	2 (3.9%)	7 (13.8%)	
Total		28 (54.9%)	23 (45.1%)	51	

Percentage of numbers of cases is showed in parenthesis. The total number of cases is n=51. Analysis is determined using a Chi Square Test and descriptive statistics. Abbreviations: CF: Female cause; CM: Male cause; MIX: Mixed cause; UK: Unknown cause.

STUDY OF THE EFFECT OF ESPERMATOZOA MOTILITY ON PREGNANCY

The main factor that was desired to evaluate, is how quality of sperm, specifically the motility, affects getting a positive or negative pregnancy. To assess the motility, data of pre and post capacitation cycles from the last insemination were collected, whether positive or negative frequency. To investigate deeply, the number of any type of spermatozoa was also collected.

In *Table 13*, summarized pre-capacitation study, before making the swim-up technique, no statistically significant results ($p>0.05$) were detected.

Table 13. Pre-capacitation motilities related to positive and negative pregnancy.

PRE-CAPACITATION			
VARIABLE	P (n=23)	N (n=28)	*p*
CONCENTRATION	78.91 ± 71.04	60.66 ± 41.49	0.564
MOTILITY	56.75 ± 18.28	54.01 ± 16.08	0.501
TYPE A	18.95 ± 17.47	16.60 ± 14.52	0.857
TYPE B	28.13 ± 13.65	23.12 ± 17.28	0.211
TYPE C	10.10 ± 5.37	14.28 ± 9.41	0.105
TYPE D	42.81 ± 17.65	45.99 ± 16.08	0.449

Comparison of means between positive and negative pregnancy with the pre-capacitation motility. Continuous variables are expressed as mean ± SD. Comparison of mean motilities ± SD which is expressed in percentage. Analysis is determined using a non-parametric Mann-Whitney U test. Abbreviations: SD: Standard deviation; P: positive; N: negative.

Regarding the post-capacitation motilities, analyse are of more importance against the pre-capacitation motilities because the swim-up has already been done and the seminal sample is introduced intrauterine to the woman and will be of great importance to get a positive pregnancy, show in *Table 14*. Type A of spermatozoa is statistically significant with positive frequency (p=0,003), in positive n=23 (91.13 ± 8.74) and in negative n=28 (78.57 ± 17.37).

Table 14. Post-capacitation motilities related to positive and negative pregnancy.

POST-CAPACITATION			
VARIABLE	**P (n=23)**	**N (n=28)**	***p***
CONCENTRATION	62.56 ± 48.78	67.32 ± 85.60	0.564
MOTILITY	98.26 ± 3.64	94.20 ± 9.33	0.230
TYPE A	91.13 ± 8.74	78.57 ± 17.37	0.003
TYPE B	4.45 ± 5.50	7.94 ± 17.37	0.123
TYPE C	2.65 ± 2.93	7.70 ± 7.49	0.001
TYPE D	1.04 ± 2.49	5.79 ± 9.33	0.090

Comparison of means between positive and negative pregnancy with the post-capacitation motility. Continuous variables are expressed as mean ± SD. Comparison of mean motilities ± SD which is expressed in percentage. Analysis is determined using a non-parametric Mann-Whitney U test. Abbreviations: SD: Standard deviation; P: positive; N: negative.

In both pre and post capacitation analyses, the concentration wasn't statistically significant (p>0.05).

To assess whether differences in pre or post capacitation affect the pregnancy result, a new variable was used using the motilities of pre and post capacitation. Motility of the pre was subtracted from the post and the differences were evaluated, see in *Table 15*. The results didn't show anything statistically significant in positive and negative pregnancies.

Table 15. Differences with pre and post capacitation motilities related to positive and negative pregnancy.

POST-PRE MOTILITIES			
VARIABLE	**P (n=23)**	**N (n=28)**	***p***
MOTILITY	41.50 ± 18.42	40.19 ± 16.35	0.798
TYPE A	72.17 ± 18.01	61.96 ± 19.10	0.077
TYPE B	-23.56 ± 15.29	-15.17 ± 20.48	0.058
TYPE C	-7.44 ± 5.29	-6.58 ± 10.36	0.719
TYPE D	41.76 ± 18.20	40.19 ± 16.34	0.649

Comparison of means between positive and negative pregnancy with the differences motilities (post-pre capacitation). Continuous variables are expressed as mean ± SD. Comparison of mean motilities ± SD which is expressed in percentage. Analysis is determined using a non-parametric Mann-Whitney U test. Abbreviations: SD: Standard deviation; P: positive; N: negative.

IMPORTANCE OF CYCLE NUMBER WITH THE PROBABILITY OF PREGNANCY

To assess a putative involvement of cycle number and the possibility to get pregnant, the number of cycle of the last insemination of each couple done in the AIH program were collected, either positive or negative pregnancy. This analysis is made to see the importance of becoming pregnant in early cycles to know if there is a relationship between positive or negative pregnancy with early or late cycles. Early cycles show a statistically significant increase in positive pregnancies (p=0.014), shows in *Table 16*.

Table 16. Relationship between the final cycle with positive or negative pregnancies.

VARIABLE	P (n=23)	N (n=28)	*p*
CYCLE	3.13 ± 1.76	4.39 ± 1,50	0.014

Cycle values range from 1 to 6. Continuous variables are expressed as mean ± SD. Comparison of mean motilities ± SD which is expressed in percentage. Analysis is determined using a non-parametric Mann-Whitney U test. Abbreviations: SD: Standard deviation; P: positive; N: negative.

STUDY OF FACTORS THAT MAY AFFECT THE MOTILITY

Until now, the effects of different factors to the pregnancy were studied. To examine if any factors can affect the motility and indirectly the pregnancy, a correlation study of biochemical and anthropological factors versus all pre motilities was performed. As can be seen in **Table 17**, not one of the correlations is statistically significant (p>0.05).

Table 17. Correlation of age and biochemical factors to motility.

PRE-CAPACITATION		
Variable	**Correlation coefficient**	***p***
AGE	-0.071	0.622
CHOLESTEROL	0.056	0.698
GLUCOSE	0.147	0.302
TAG	-0.057	0.693
SEMINOGRAM		
Variable	**Correlation coefficient**	***p***
AGE	-0.156	0.275
CHOLESTEROL	-0.135	0.345
GLUCOSE	0.074	0.607
TAG	-0.017	0.905

Analysis is determined using a Spearman Bivariate Correlation.. Abbreviations: TAG: triacylglycerol

Also, the influence of snuff on the motility was compared using a Mann-Whitney U analysis and nothing statistically significant was observed in any case (*p*>0.05).

MULTIVARIATE ANALYSIS (MV)

In multivariate analysis adjusted for age of male and female and cause of infertility (CF, CM, MIX, UK) the statistically significant results obtained above were studied. To know if statistically significant results are associated independently with other variables.

These variables were the motility of type a spermatozoa in post capacitation, the number of cycles and the female's triacylglycerol's.

- The motility of the type a spermatozoa in post-capacitation associated independently with positive pregnancy(p=0.031 B=0.91; Exp(B)=1.095 [1.008-1.189]).

- Early number of cycles associated independently with positive pregnancy (p=0.014 B=(-)0.497; Exp(B)=0.608 [0.409-0.905]).
- The increase of female triacylglycerol's associated independently with negative pregnancy (p=0.035 B=(-)0.014; Exp(B)=0.986 [0.973-0.99]).

Although motility pre and post capacitation didn't give statistically significant results, they were added to the MV because they will be interesting for future investigations with more couples. In any of the cases of pre or post total motility a statistically significant value can be observed.

Table 18. Logistical regression of statistically significant values and post/pre capacitation motilities.

VARIABLE	p	B	Exp (B)	OR	
				Lower	Higher
Post A	0.031	0.91	1.095	1.008	1.189
TAGF	0.035	-0,014	0.986	0.973	0.990
Cycle	0.014	-0.497	0.608	0.409	0.905
Mobpost	0.799	-0.22	0.978	0.827	1.158
Mobpre	0.699	-0.009	0.991	0.949	1.036

Analysis is determined using a multivariate logistic regression. The OR is a main way to quantify how strongly the presence or absence of property A is associated with the presence or absence of property B, similarity to risk ratio. Abbreviations: Post A: spermatozoa type A of post-capacitation; TAGF: female triacylglycerol's; Mobpost: Post-capacitation motilities: Mobpre: Pre-capacitation motilities; OR: Odds ratio.

5. DISCUSSION

It is estimated that infertility affects an estimated of 70 to 80 million couples worldwide and that 15% of those living in developed countries would go for a consultation for it[38]. One of the main factors in the female reproductive capacity decline is the incipient delayed childbearing[39]. In fact, in the Joan XXIII University Hospital Laboratory a mean age in 2008 of 32.4 years old and a mean age in 2014 of 34.1 years old has been observed. The introduction of assisted reproduction techniques (ART) has increased the chances of effective treatment, becoming a basic option for infertile couples, one of these options being the AIH.

Earlier this century, Goverde stated that the age of the woman is the most influential factor in the probability of pregnancy, whatever the chosen reproductive treatment. Given the existing literature, it is known that female fertility peaks between 20 and 24 years, then decreases uniformly, going down significantly from 35 years and very marked way past 40[12,39]. If we refer to our results, no significant differences were observed, even when studying women we can see that the average is lower in the negative than in the positive results (*Table 10*). This value was not what we had been waiting for, and could be because of the low amount of couples participating in the study.

As reflected in *Table 9*, reproductive capacity is clearly affected by different types of causes and pathologies. Female cause is the most common with a 43.1% and the most common diagnostic within this group is POS. The small number of samples doesn't allow to do a significant analysis but our result is related with other articles where they have done the study with POS[40-43] in which remarked that POS is the most prevalent endocrinopathy in young women, affecting 4-8% of the females at childbearing age population. It's a multifactorial pathogenesis, which may be treated; this would be an interesting aspect prior to *AIH.* Although nothing statistically significance exists in our results, there is a trend of negative pregnancy result when the cause is feminine, and a more positive trend when the cause is masculine, mixed or unknown (*Table 12*), this makes us think that the treatment (*AIH*) performed on women based on improving their pathology, like POS, could be improved.

The main purpose of *AIH* is to achieve pregnancy. This is why so many different biochemical (TAG, glucose and cholesterol) and anthropological (age and snuff) factors were analysed, taking into account the pregnancy result. With regard to glucose, cholesterol, age and snuff statistically significant results weren't obtained but if we consult

the literature we can see that there is a relationship between snuff and age with fertility[3,7,11], this aspect need not alter the final result in the *AIH* technique. Therefore, the increase of female triacylglycerol's associated independently with negative frequency (*p*=0.035). The results indicate that an increased 1 mg/dL of TAG in female, produces a decrease of 1.4% in the probability of pregnancy (*Table 10; Table 18*). Results indicate that TAG lower than 140 mg/dL is related with positive pregnancy. Although the average of female TAG in negative pregnancy is within the normal range, known to ovulatory capacity decrease with more TAG[44]. One of the possible treatments before starting the cycles would be to go on a diet to lower the number of TAG in women.

To determine the relationship between sperm motility parameters and *AIH* pregnancy rates, pre and post capacitation semen samples were analysed (*Table 13, Table 14*). Significant positive correlations were observed between post capacitation percent progressive motility (type a) and pregnancy (p=0.031 B=0.91). The statistic analysis confirms that the statistical significance was independently associated with pregnancy rates and obtained that when progressive sperm increases 1%, there is a 9.5% higher probability of positive pregnancy (Exp(B)=0.095) (*Table 18*). We could say that more than 90% of type a of spermatozoa increases the probability of positive pregnancy. This correlates with other studies[45,46]. This may be due to the great difference that exists between type a of spermatozoa and the others (b and c). Type a of spermatozoa are those which swim forward and fast in a straight line, and so it is easier to get into the ovule avoiding insemination problems.

One of the negative forecasts towards pregnancy is the number of cycles. This stipulated that the maximum number of cycles is six and from four, technique loses success probability, as stated by the SEF. One of the important results of the work, has been the analysis of the relationship between the last cycle carried out by each couple and the frequency to get pregnant. Statistical analysis demonstrated that early number of cycles associated independently with positive frequency or inversely proportional, latest number of cycles associated independently with negatives (*p*=0.014) (*Table 18*). One of the interpretations we can do is that when the couple take more than four cycles the probability of pregnancy is reduced to 39.2% (Exp(B)=0.608).

Regarding the study, if they had factors affecting sperm motility, nothing of statistical significance was obtained (*Table 17*). Other studies show that factors we have studied affect sperm motility such as snuff or TAG[44]. Other factors that may affect sperm motility

are antidepressants because they appear to be one of the side effects of paroxetine, one of the most common antidepressants, and this is to slow the sperm. One of the possible treatments before sperm capacitation would be a diet with B12 vitamin, zinc and Q10 Coenzyme.

Scientific studies have shown a direct relationship between vitamin B12 and sperm motility. It was revealed that B12 vitamins act as an antioxidant and reduce oxidative stress in sperm cells. Oxidative stress can change the structure of sperm cells and reduce sperm motility. This study demonstrates that a greater amount of vitamin B12 can reduce stress oxidative risk and this increases sperm motility[47].

Another scientific study has also shown that adequate levels of coenzyme Q10 are beneficial for sperm motility. This study was carried out by the Obstetrics and Gynaecology Department of the Medicine Faculty in Israel University who found that oral administration of Coenzyme Q10 had a significant increase in motility of study subjects. The result of this study predicts the positive effect of Coenzyme Q10 on sperm function and motility[48].

These possible treatments along with a healthy diet and life may improve sperm motility in males before sperm capacitation and therefore to obtain better results.

Finally, one of the interesting comparisons is that of Joan XXIII University Hospital positive/cycle% versus SEF data. Our results are slightly lower than the SEF, this may be because we are a small hospital with fewer samples than other hospitals. It was also evaluated by SEF. Still obtaining fewer results compared to SEF, in the last years there has been an increase in positive/cycle% of results (5.56% in 2009; 8.57% in 2010; 10% in 2011; 10% in 2012). SEF (12.6% in 2009; 12.9% in 2010; 13.9% in 2011; in 2012) The information from 2012 and 2013 still isn't available. (*Informes_Registre_SEF)*

6. CONCLUSIONS

1. Female causes are the most common of infertility causes in our results and the major cause of female infertility is POS. Other factors like age are very important in women, in theory not so much in men.

2. After performing sperm capacitation (swim-up) all the sperm samples have a sizeable increase in their motility, showing statistically significant difference between positive or negative pregnancies. This difference is clearly apparent in spermatozoa type a or PM. Maintaining type a spermatozoa levels up to a 90% increase in the chances of pregnancy.

3. When comparing the number of cycles with the pregnancy frequency it is corroborated that most pregnancies acquired by AIH are achieved within the first four cycles. Pregnancy rates are significantly reduced from the fourth cycle.

4. The analysis of TAG regarding pregnancy concludes that maintaining levels of female TAG below 140 mg/dL increases pregnancy probabilities and by treating it we can raise the success of pregnancy.

5. We can conclude that improving some aspects in males and females using different treatments, like increase the mobility of type a spermatozoa, could improve the effectiveness of the technique and therefore have more chance of reaching our objective, the pregnancy.

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SUPPLEMENTARY MATERIAL

ANNEX 1: SEMINAL PAPER

Institut Català de la Salut Laboratori Clínic ICS Camp de Tarragona	IMPUR-009 ESPERMIOGRAMAS	
Data	Responsable :	Página 1 de 1

rev.02

Fecha :

Nombre :

Diagnóstico :

Aspecto :

Licuefacción :

Viscosidad :

Volumen : mL

pH :

Movilidad a + b : %
c
d

Viabilidad : %

Aglutinación :

Agregación :

Antic. antiesperm. :

Concentración : esperm./mL

Recuento total : esperm.

Formas normales : %

Células redondas :

ANNEX 2: REAL NORMAL AND ABNORMAL MORPHOLOGY

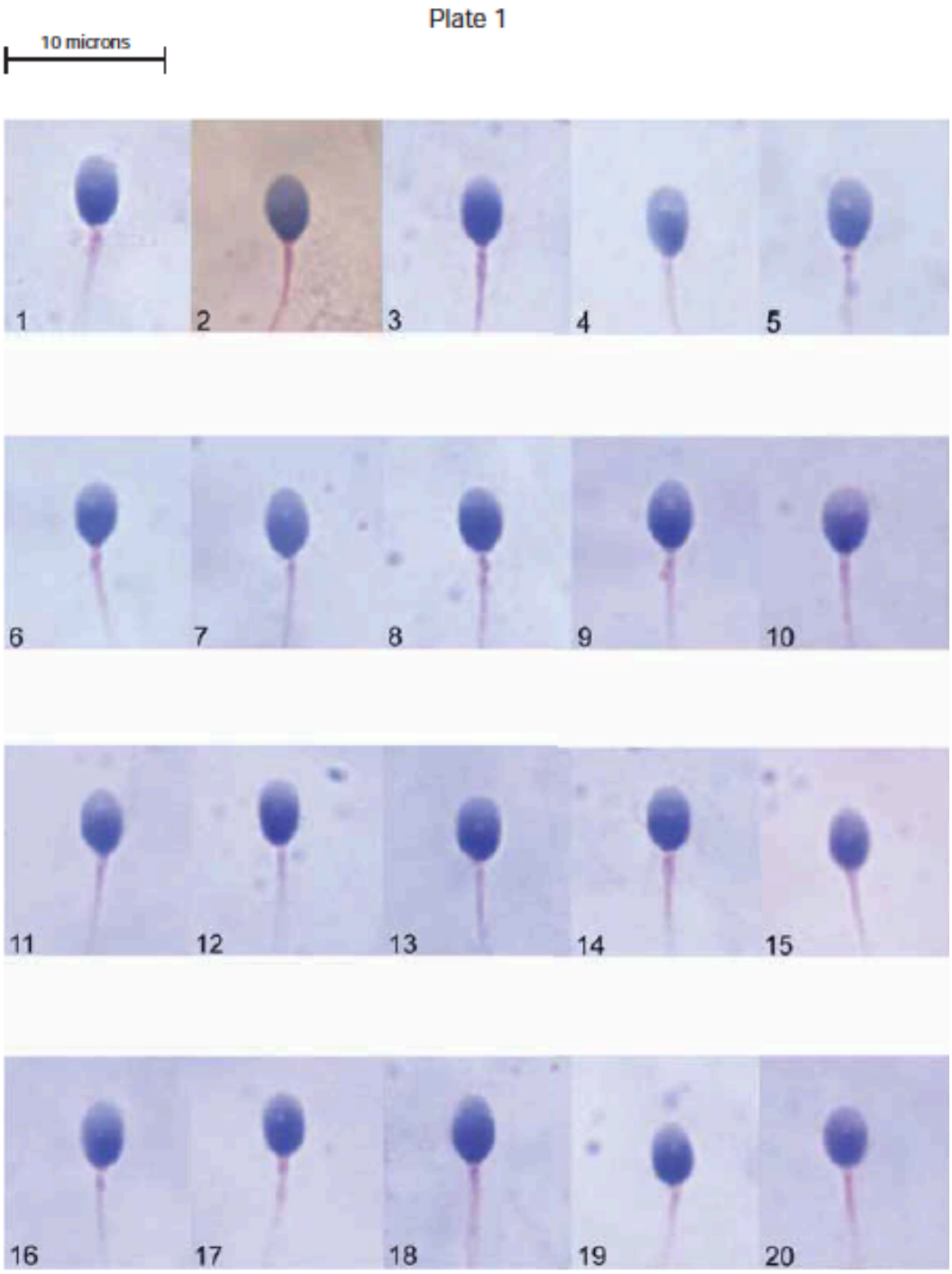

Fig 5. Plate 1 with all normal forms of sperm, explained in table 3

Table 3. Morphology assesment of spermatozoa in Plate 1.

Sperm	Head shape	Other head comments	Midpiece comments	Principal piece comments	Overall sperm classification	Comments
1	normal		normal		normal	if PP OK
2	normal		normal		normal	if PP OK
3	normal		normal		normal	if PP OK
4	normal		normal		normal	if PP OK
5	normal		normal		normal	if PP OK
6	normal		normal		normal	if PP OK
7	normal		normal		normal	if PP OK
8	normal		normal		normal	if PP OK
9	normal		normal		normal	if PP OK
10	normal		normal		normal	if PP OK
11	normal		normal		normal	if PP OK
12	normal		normal		normal	if PP OK
13	normal		normal		normal	if PP OK
14	normal		normal		normal	if PP OK
15	normal		normal		normal	if PP OK
16	normal		normal		normal	if PP OK
17	normal		normal		normal	if PP OK
18	normal		normal		normal	if PP OK
19	normal		normal		normal	if PP OK
20	normal		normal		normal	if PP OK

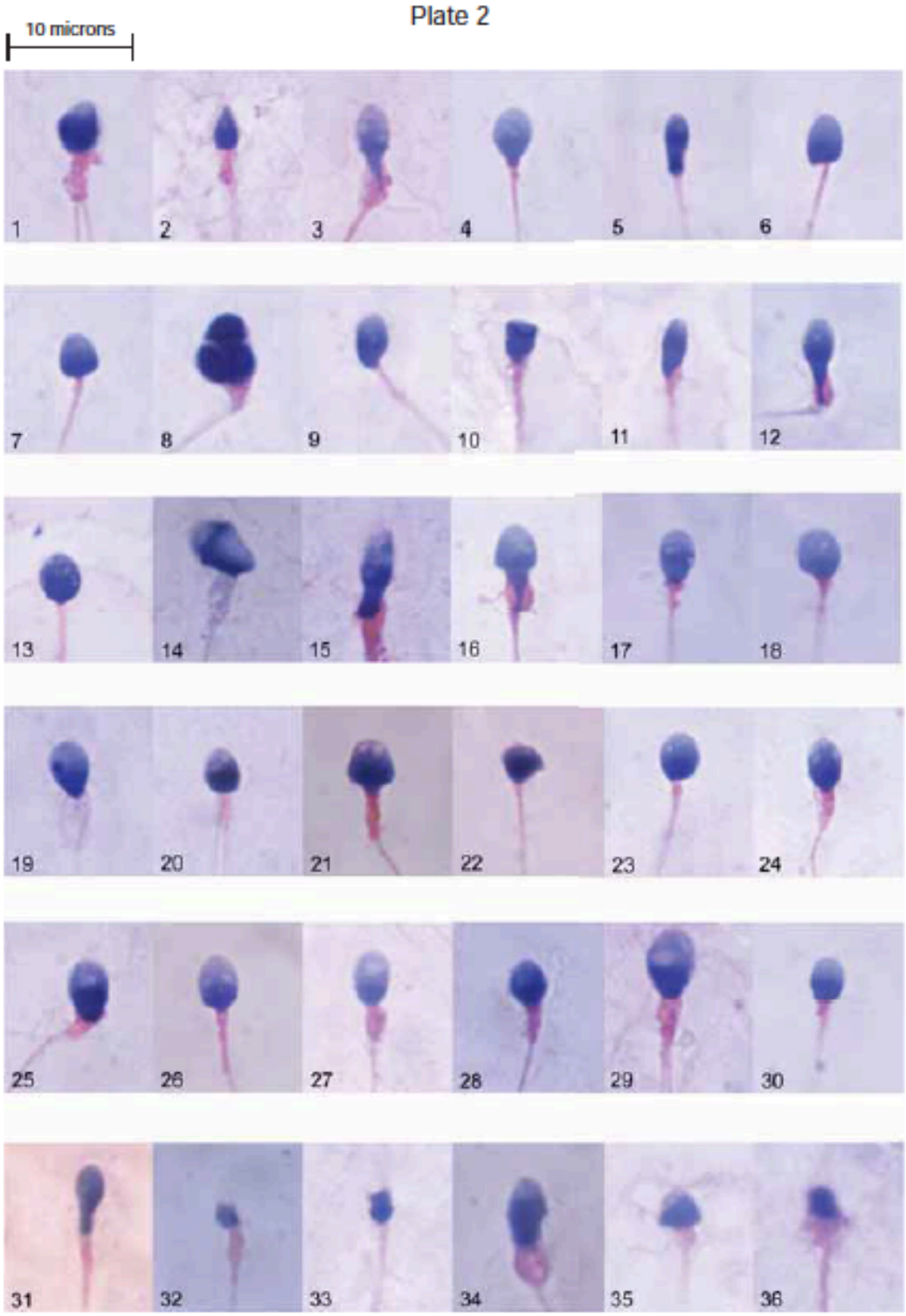

Fig 6. Plate 2 with normal and abnormal forms of spermatozoa, explained in table 4.

Table 4. Morphology assesment of spermatozoa in Plate 2.

Sperm	Head shape	Other head comments	Midpiece comments	Principal piece comments	Overall sperm classification	Comments
1	abnormal		thick	double	abnormal	
2	abnormal		irreg		abnormal	side view
3	abnormal	pyriform	bent, irreg, ERC		abnormal	>one third
4	abnormal				abnormal	
5	abnormal	pyriform			abnormal	
6	abnormal				abnormal	
7	abnormal				abnormal	
8	abnormal		thick		abnormal	
9	abnormal		insert		abnormal	
10	abnormal				abnormal	
11	abnormal				abnormal	
12	abnormal	pyriform		bent	abnormal	
13	abnormal	>2 vac, PA vac			abnormal	
14	abnormal		thick		abnormal	
15	abnormal	pyriform	thick, ERC		abnormal	>one third
16	abnormal	pyriform	ERC		abnormal	>one third
17	normal	PA vac			abnormal	
18	abnormal		thick, insert		abnormal	
19	abnormal		abnormal		abnormal	
20	abnormal		thick		abnormal	
21	abnormal		thick		abnormal	
22	abnormal				abnormal	
23	abnormal				abnormal	
24	normal	>2 vac	thick		abnormal	
25	abnormal		thick, bent		abnormal	
26	abnormal		thick		abnormal	
27	abnormal	>70% acr	thick		abnormal	
28	abnormal		thick		abnormal	
29	abnormal		thick		abnormal	
30	abnormal		thick		abnormal	
31	abnormal	pyriform	thick		abnormal	
32	abnormal	small	thick		abnormal	
33	abnormal	small	thick		abnormal	
34	abnormal		ERC		abnormal	>one third
35	abnormal		thick		abnormal	
36	abnormal		thick		abnormal	

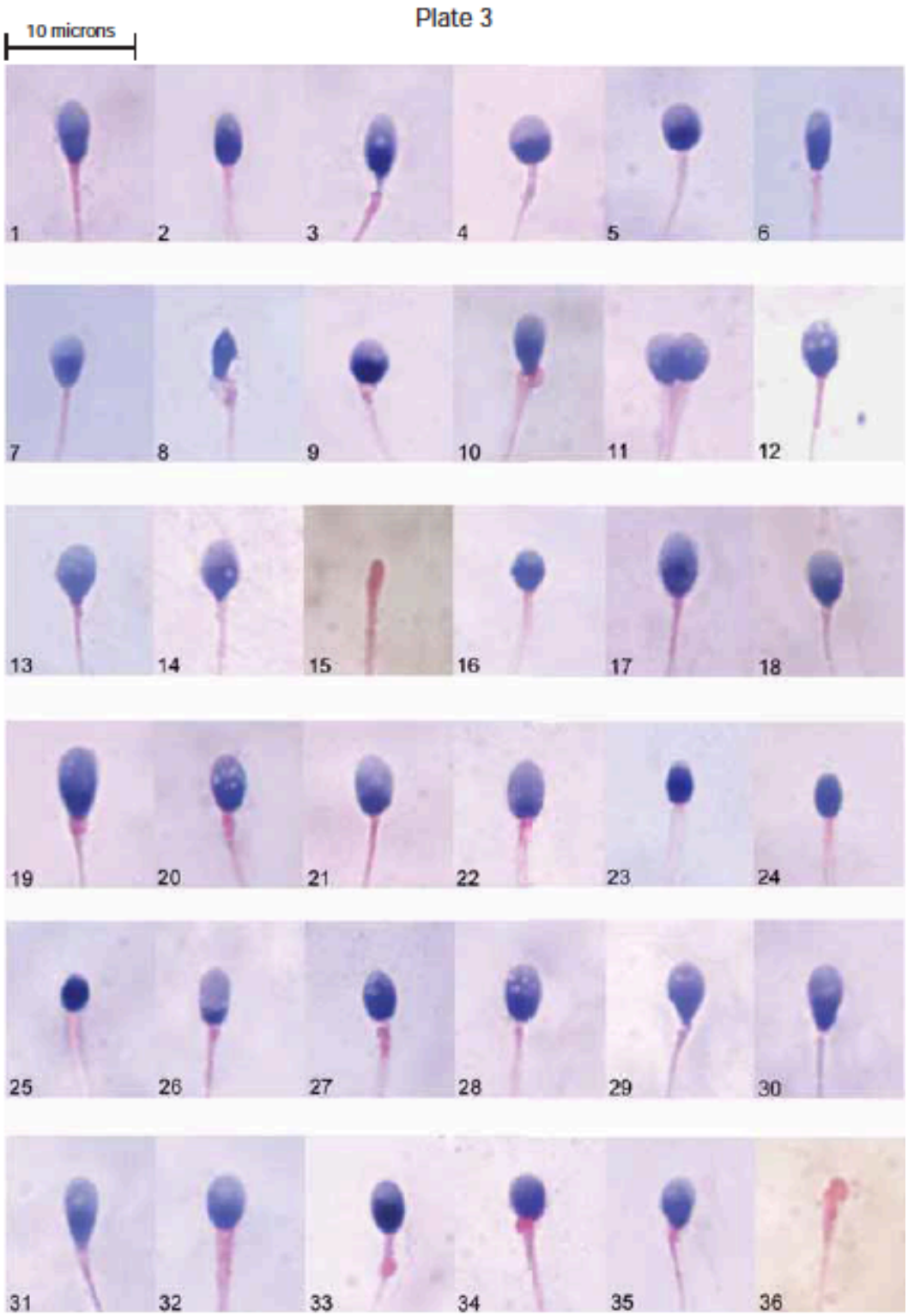

Fig 7. Plate 3 wih normal and abnormal forms of spermatozoa, explained in table 5.

Table 5. Morphology assesment of spermatozoa in Plate 3.

Sperm	Head shape	Other head comments	Midpiece comments	Principal piece comments	Overall sperm classification	Comments
1	abnormal	tapered	thick		abnormal	
2	abnormal				abnormal	
3	abnormal		irreg		abnormal	
4	abnormal	round			abnormal	
5	abnormal	round			abnormal	
6	abnormal	tapered			abnormal	
7	abnormal	tapered			abnormal	
8	abnormal	amorphous	thick		abnormal	
9	abnormal	round	thick		abnormal	
10	abnormal	tapered	irreg, thick		abnormal	
11	—				—	two cells
12	abnormal	>2 vac, PA vac			abnormal	
13	abnormal				abnormal	
14	normal	PA vac			abnormal	
15	—				—	pinhead
16	abnormal	small			abnormal	
17	abnormal	large			abnormal	
18	normal		thick		abnormal	
19	abnormal		thick		abnormal	
20	abnormal	>2 vac	insert		abnormal	
21	normal	>70% acr			abnormal	
22	abnormal	>70% acr			abnormal	
23	abnormal	<40% acr, small			abnormal	
24	abnormal	<40% acr. small			abnormal	
25	abnormal	<40% acr, small			abnormal	
26	abnormal	>70% acr			abnormal	
27	abnormal	<40% acr, >2 vac	irreg		abnormal	
28	normal	>2 vac			abnormal	
29	abnormal	tapered			abnormal	
30	abnormal	tapered			abnormal	
31	abnormal	tapered			abnormal	
32	normal		thick		abnormal	
33	normal		thick		abnormal	
34	abnormal	<40% acr	thick		abnormal	
35	abnormal		thick, bent		abnormal	
36	—				—	pinhead

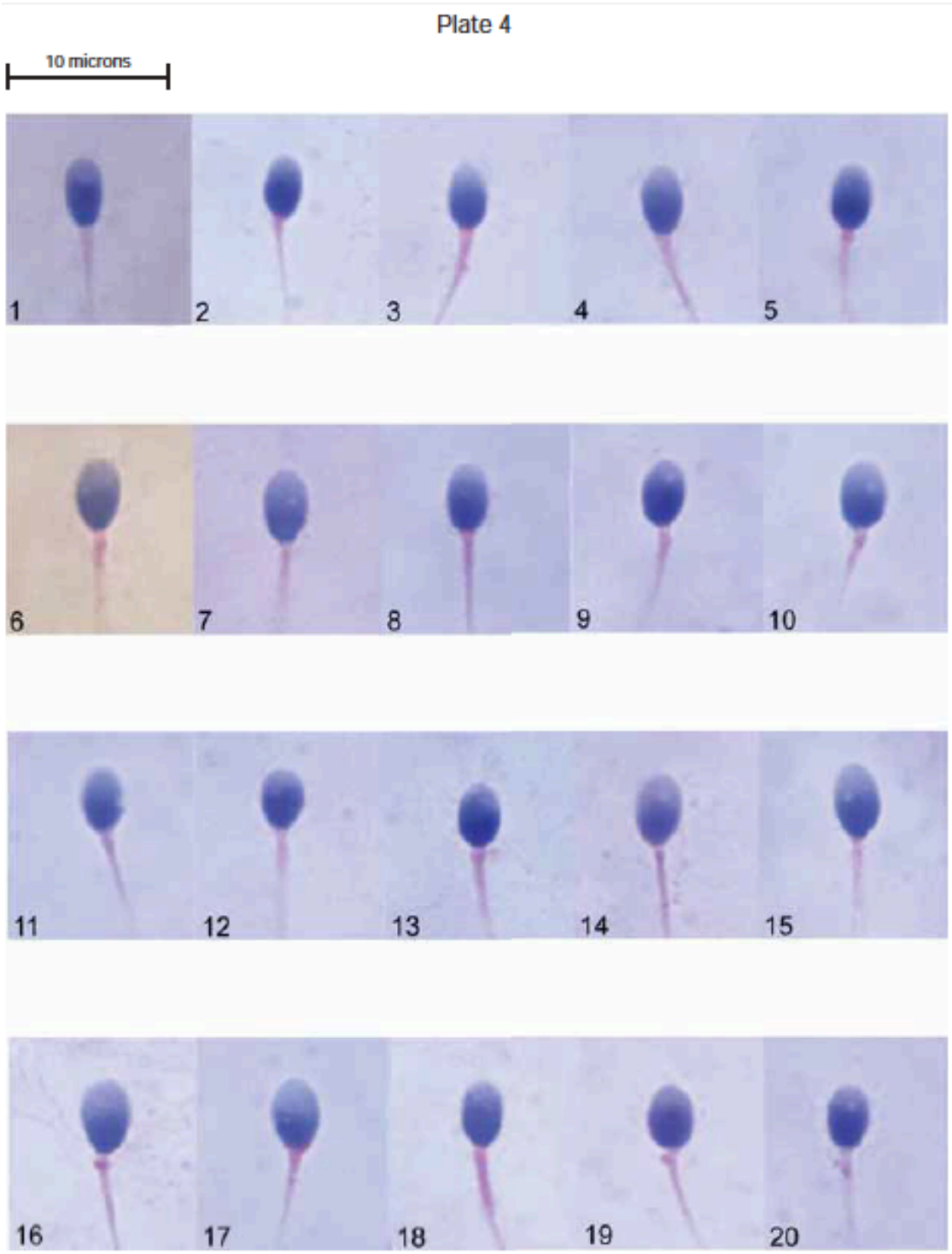

Fig 8. Plate 4 with normal and abnormal forms of spermatozoa, explained in Table 6.

Table 6. Morphology assesment of spermatozoa in Plate 4.

Sperm	Head shape	Other head comments	Midpiece comments	Principal piece comments	Overall sperm classification	Comments
1	abnormal	flat	thick		abnormal	
2	normal		thick, bent		abnormal	
3	normal		thick		abnormal	
4	normal		thick, bent		abnormal	
5	normal		thick		abnormal	
6	normal		thick		abnormal	
7	abnormal	irreg			abnormal	
8	normal		thick		abnormal	
9	normal		insert, bent		abnormal	
10	normal		thick, bent		abnormal	
11	abnormal	PA vac			abnormal	
12	abnormal				abnormal	
13	abnormal	<40% acr, >2 vac	thick		abnormal	
14	normal		irreg		abnormal	
15	normal		insert		abnormal	
16	normal		thick		abnormal	
17	normal		insert, thick		abnormal	
18	normal		thick, too long		abnormal	
19	normal	<40% acr	insert		abnormal	
20	normal	<40% acr	irreg		abnormal	

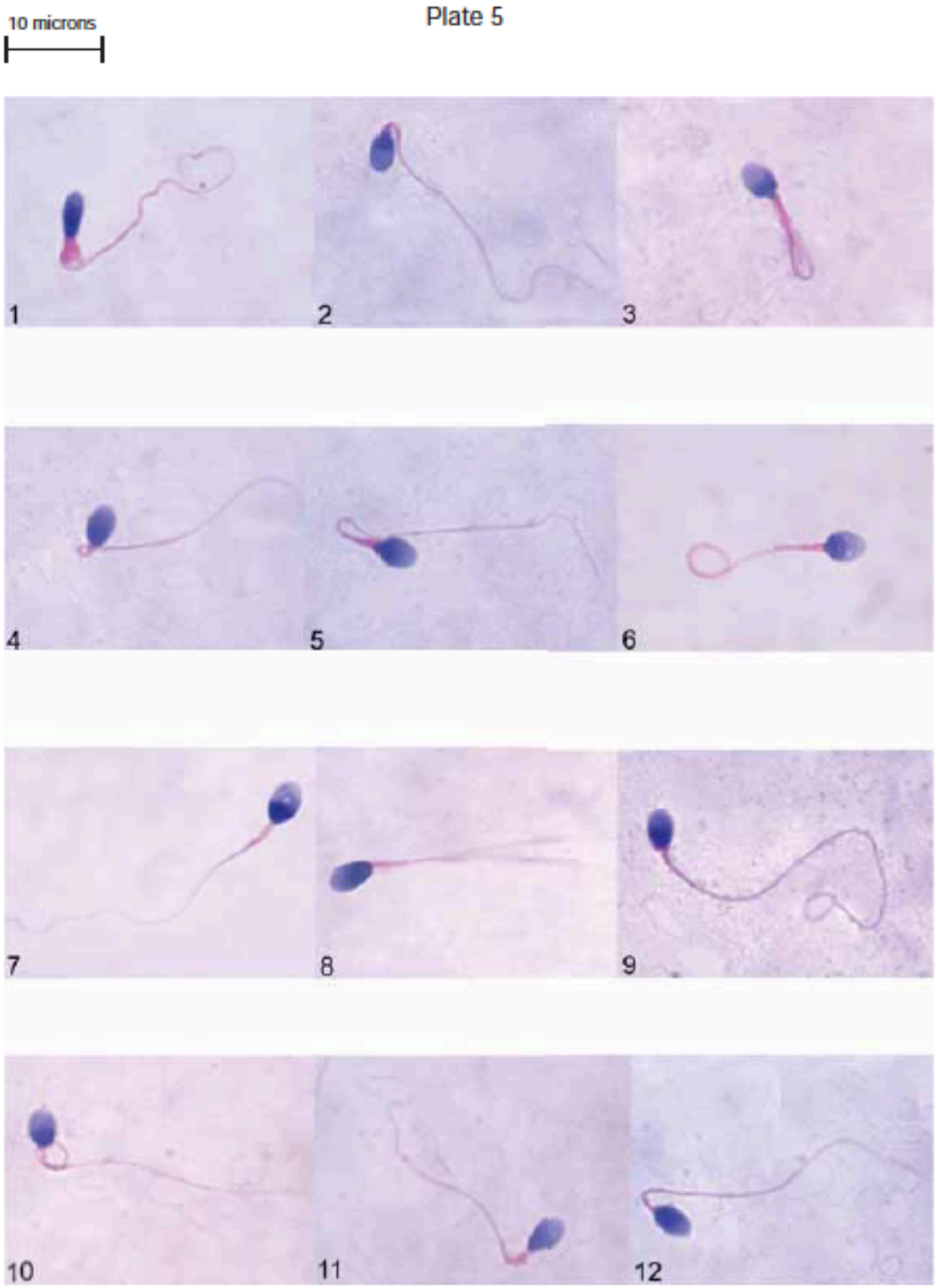

Fig 9. Plate 5 with normal and abnormal forms of spermatozoa, explained in table 7.

Table 7. Morphology assesment of spermatozoa in Plate 5.

Sperm	Head shape	Other head comments	Midpiece comments	Principal piece comments	Overall sperm classification	Comments
1	abnormal		ERC		abnormal	>one third
2	normal		bent	normal	abnormal	
3	abnormal	>70% acr		looped	abnormal	
4	normal		bent	normal	abnormal	
5	normal		thick	looped	abnormal	
6	abnormal	PA vac		coiled	abnormal	
7	normal				normal	
8	normal			double	abnormal	
9	abnormal			coiled	abnormal	
10	abnormal		bent, insert	coiled	abnormal	
11	normal		thick	bent	abnormal	
12	normal		bent	normal	abnormal	

ANNEX 3: AIH PAPER

Institut Català de la Salut Laboratori Clínic ICS Camp de Tarragona	IMPUR-010 CAPACITACIO ESPERMATICA	
Data	Responsable :	Pàgina 1 de 1

rev.02

DONA

NHC :

NOM:

DATA:

HOME

NHC:

NOM:

DATA:

INDICACIO

FIV | IAC | IAD | ICSI | REM

SEMEN FRESC

HORA DE RECOLLIDA

DIES D'ABSTINENCIA

VOLUM	REC (x10^6)	MOVILITAT	GRAU	VISCOSITAT	AGLUTINACIO

Observacions:

SEMEN CAPACITAT

VOLUM	REC (x10^6)	MOVILITAT	GRAU	REM(x10^6)/ml	REMT (x10^6)

Dilució del semen: 1/..... REM (dilució): ..... x 10^6

CONCENTRACIO ESPERMATOZOOS / OVOCITS

VOLUM INSEMINAT

ABBREVIATIONS

AB – Antibody

AI – Artificial insemination

AID – Artificial insemination with donor's semen

AIH – Artificial insemination with husband's semen

CF – Cause feminine

CM – Cause masculine

DNA – Deoxyribonucleic acid

E_2 – Stradiol

Fig – Figure

FSH – Follicle-Stimulating Hormone

GnRH – Gonadotropin-Relasing Hormone

HBC – Hepatitis B Virus

HCG – Human Chorionic Gonadotropin

HCV – Hepatitis C Virus

HIV – Human Immunodeficiency Virus Infection

IM – Immobility

IUI – Intrauterine Insemination

LH – Lutenizing Hormone

MIX – Mixed cause

mL – Mililiters

NP – Non-progressive motility

OSC – Ovarian Controlled Stimulation

PR – Progressive motility

RPR – Fast plasmatic regain

SCA – Sperm Class Analyzer

SD – Standard deviation

SEF – Sociedad Española de Fertilidad

SIT – Sexual Transmitted Infection

POS – Polycystic Ovary Syndrome

TAG – Triacylglycerol

TSH – Thyroid-Stimulating Hormone

WHO – World Health Organization.