Dipartimento di Medicina Molecolare e dello Sviluppo

Dottorato Di Ricerca In Medicina Molecolare

Ciclo XXXIV

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Selection of competent oocytes by morphological features. Can an artificial intelligence - based model predict oocyte quality?

Settore Scientifico-Disciplinare: BIO/13, BIOLOGIA APPLICATA

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Firmato il 17/06/2022

ANNO ACCADEMICO: 2020/2021

INTRODUCTION

1 BACKGROUND

Infertility is a wide medical and social problem.

The World Health Organization (WHO) defines infertility as a failure to achieve clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (World Health Organization et al., 2018). Infertility affects millions of people worldwide and in Italy is estimated to affect about 15% of women and men of reproductive age. Infertility has a direct impact on affected people and their family, but, due to its high prevalence, it represents a growing economic and societal challenge.

Infertility affects both men and women, and in about half of all cases, infertility is caused by female-related factors. Female infertility is classified as primary or secondary.

Primary, when a woman was never pregnant and can't conceive after one year of not using birth control.

Secondary: if a woman was unable to establish again a clinical pregnancy despite one previous successful pregnancy. The same categorization might be applicable to the male regarding his participation in the initiation of a pregnancy.

The three major factors affecting the spontaneous probability of conception are (a) time of unwanted non-conception (b) age of the female partner and (c) disease-related infertility (Gnoth et al., 2005). Semen decline that has been observed over time, endocrine disrupting chemicals and consanguinity are other factors that may be involved.

a) ***Time of unwanted non-conception***: the main cause affecting the individual spontaneous pregnancy prospect is the time of unwanted non-conception, which determines the severity of subfertility. 80% of the pregnancies occur in the first six cycles with regular intercourse in the fertile period. One out of two couples of the residual twenty percent couples without conception will conceive spontaneously in the next six cycles (Gnoth et al., 2005)

b). ***Female fertility decline:*** The association between female education and age at becoming a parent is well-documented. Early studies demonstrated a strong inverse relationship between education and fertility, with education impacting the timing of first births (Martin, 2000). Women decide to continue schooling and acquire a profession before thinking of having children with as a consequence a significant postponement of childbearing in Western

societies (Lutz et al., 2003). Currently, the mean maternal age at first birth is approaching 30 years in several European countries and many women deliver their first child at age 35 or older (Eijkemans et al., 2014). The problem arising with delayed child wish is that the fertility decline already starts around 25-30 years of age. This suggests that there is a fairly universal pattern of age-related fertility decline. The peak of "fertility" is reached at around 20-24 years of age, after which a slow and steady decline begins that becomes much more rapid between the ages of 38 and 40 (Broekmans et al., 2009; Hart, 2016). Reaching the age of 40 years of age may itself represent the primary cause of female infertility (Figure 1)

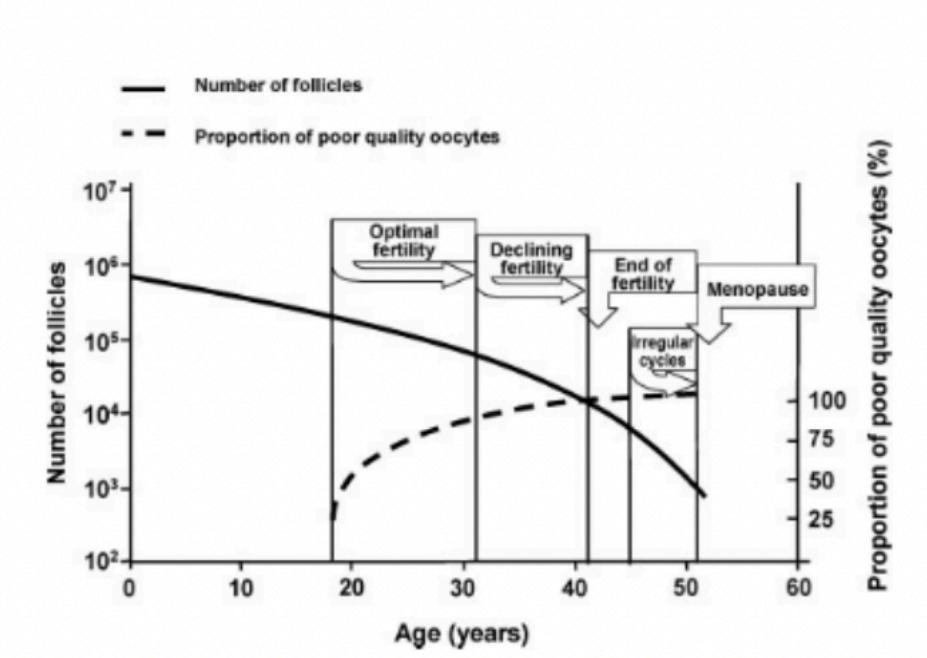

Figure 1. *Schematic representation of the number of primordial follicles present in the ovaries and the chromosomal quality of oocytes in relation to female age and corresponding reproductive events (Broekmans et al., 2009).*

At age 30, the probability for a woman to achieve pregnancy is, each month, 20%, a percentage that drops dramatically to 5% after the age of 35. These values are valid both for natural conception and in the case of recourse to techniques of medically assisted procreation. The literature reports that the fecundity of women decreases gradually but significantly beginning approximately at age 32 years and decreases more rapidly after age 37 years, (American College of Obstetricians and Gynecologists Committee on Gynecologic Practice and Practice Committee, 2014) but it should be considered that the decline can follow physiologically different trends from woman to woman, because each woman at birth has a different and predefined number of egg cells that can decrease more or less quickly before and/or after menarche.

The age of the woman does not only influence the number of oocytes but also their quality which seems to be closely related to the presence of aneuploidies (Tatone et al., 2008); The rate of oocyte aneuploidy is lower for women by up to age 35 (53 % in embryos after 3 days from fertilization), but increases to 74 % at the age of 41–42, and to 93 % after the age of 42 (Harton et al., 2013; Meczekalski et al., 2016).

Often, however, the biological age does not correspond to the chronological age and for this reason hormonal tests can be performed to determine the residual fertility (Broekmans et al., 2006). This evaluation consists of the dosage of anti-Müllerian hormone and the count of antral follicles through an ultrasound performed between the 2nd and 4th day of the cycle.

The combination of these two tests can reliably predict the possibility of pregnancy and the course of the cycle.

A woman with a reduced ovarian reserve has less chance of becoming pregnant than a woman of the same age with a greater ovarian reserve. It should be noted that reduced ovarian reserve is seen not only in older women, but a number of risk factors have been reported in the literature including family history of early menopause, genetic factors (45, X chromosome mosaicism, FMR1 gene mutation, etc.), diseases that may cause ovarian damage (endometriosis, pelvic tuberculosis, pelvic infection, etc.) or history of ovarian surgery, chemotherapy for ovarian-related diseases, pelvic radiotherapy and autoimmune diseases, smoking, and environmental factors. (Rasool & Shah, 2017).

c) ***Disease-related infertility:*** it may affect both genders or be specific to one gender as summarized below (Table 1).

Both Genders	▪ Hypogonadotrophic hypogonadism
Female	▪ Premature ovarian insufficiency
Male	▪ Testicular deficiency ▪ Post-testicular impairment

Table 1. *Disease-related infertility (Vander Borght & Wyns, 2018)*

In particular, according to the data provided by the *Istituto Superiore di Sanità (ISS, 2019)*, in 21,6% of cases the male factor is predominant, in 41,9% of cases the female factor is predominant, in 19,4% there is a couple factor, i.e., a condition in which coexist male and female subfertility, and in the remaining 16,2% the etiology remains sine causa (Figure 2).

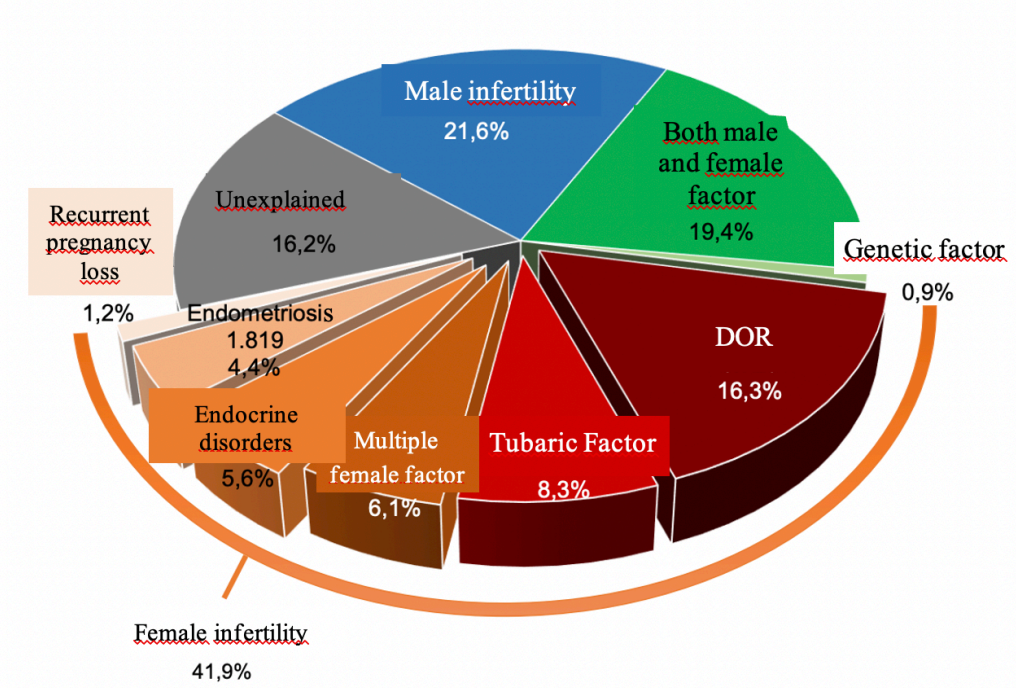

Figure 2. *Causes of infertility, data collected by the National Registry of Medically Assisted Procreation. (ISS 2019)*

2 ASSISTED REPRODUCTIVE TECHNOLOGIES (ART).

The number of treatments with ART is steadily increasing. Since the birth of the first girl conceived *in vitro* in 1978, the in vitro fertilization technique (IVF) over the years have been progressively applied worldwide and new techniques have been developed that have made it possible to increasingly broaden the indications and to increase the effectiveness of treatments. Today, the IVF include several medical treatments that can resolve many forms of infertility. Data from the various international IVF registries estimate that each year more than one million cycles are performed each year and then, thanks to these technologies, more than 7 million individuals are born.

The ART techniques are divided into basic techniques or Level I, simple, minimally invasive, involving in vivo conception, and advanced techniques of Level II and III, complex and more invasive and involving egg retrieval. The choice of the technique to be applied is made after a thorough clinical course of the couple and always in a gradual way (Forabosco, 2005)

- level I techniques, which involve "in vivo" conception
    - Targeted sexual intercourse
    - Intra-Uterine Insemination (IUI)
- level II techniques, which involve the surgical retrieval of oocytes
    - *In Vitro* Fertilization (IVF);
    - Intracytoplasmatic Sperm Injection (ICSI);

Targeted sexual intercourse

Consists, by means of ultrasound and/or blood tests, in targeting the most fertile time of the female cycle. After these tests are carried out, the doctor will inform the couple of the most favourable period of the female cycle for sexual intercourse in view of becoming pregnant.

Intra-Uterine Insemination (IUI)

Consists in the insertion into the uterine cavity, through a specific catheter, of a semen sample after appropriate preparation,

This technique can be performed on spontaneous cycle or following ovarian stimulation, and is always associated with ultrasound and hormonal monitoring. It is not invasive but the success rates are lower than those obtained with the second level techniques.

IUI is indicated in cases of idiopathic infertility, mild male factor, ovulatory disorders and mild endometriosis

In Vitro Fertilization (IVF)

The first technique developed for the infertile couple, which allowed the birth of the first girl in the world conceived with the IVF technique is the traditional IVF that consist of fertilization "in vitro" and the subsequent transfer of embryos in utero.

The main steps of the IVF are oocytes collection, their fertilization, and the subsequent transfer of the developed embryos into the maternal uterus. This technique is indicated in case of tubal pathologies, repeated failures of IUI, idiopathic sterility and moderate male factor.

Intracytoplasmatic sperm injection

ICSI was introduced in the early 1990s as one of the most dramatic technological breakthroughs in ART. After its introduction, the technique was rapidly incorporated into the routine clinical practice of fertility centers throughout the world (Rubino et al., 2016). This technique,

discovered in 1992 by the Italian researcher Gianpiero Palermo (Palermo et al., 1992), was born to solve all those cases of infertility from male factor, where there was not the possibility or sufficient number or motility of spermatozoa to attempt spontaneous fertilization. Also, in this case the meeting of the gametes takes place outside the woman's body, but with a different method of fertilization of the oocyte: it is the operator who selects and injects the single spermatozoon inside the cytoplasm, through the use of a sophisticated instrument such as the micromanipulator. After successful fertilization, the embryos are transferred into the uterus. This technique is indicated in cases of severe oligo-asthenoteratozoospermia, obstructive azoospermia and subsequent surgical recovery of spermatozoa and cryopreserved oocytes.

Infertility rate among couples gradually increases worldwide due to delaying childbearing, which is the result of various social and economic factors (Balasch & Gratacós, 2012). Thus, ARTs acquire more and more importance each day.

Even though there have been gradual improvements in the success rate, these are quite low at 35% (The European IVF-monitoring Consortium (EIM)‡ for the European Society of Human Reproduction and Embryology (ESHRE) et al., 2020); this implies that there is potential for improvement in the crucial steps in ART treatments, such as the selection of the best oocyte to inseminate.

In the everyday work of an average IVF laboratory usually morphological assessment of the retrieved oocytes is rather superficial; in the case of ICSI, a rapid evaluation using an inverted microscopy is performed after denudation, including evaluation of the cytoplasm, the perivitelline space (PVS) and the zona pellucid (ZP). This evaluation provides very superficial and approximative information about the stage of development (germinal vesicle, metaphase I or II) and the quality. Afterwards all MII phase oocytes are subjected to ICSI, and from that point the developmental potential of the obtained embryo is estimated exclusively on the basis of the morphology of the embryo. Selecting high- quality oocytes based on morphological analysis may increase success rates of fertilization and subsequent embryo development (Gilchrist et al., 2008).

The main challenge related to selecting better oocytes is that developmentally incompetent oocytes may exhibit the same morphologies as the good ones.

3 THE OOCYTE

3.1 OOGENESIS AND FOLLICOLOGENESIS

The two main function of the ovary are: the differentiation and release of the mature oocyte for fertilization and the production of steroid hormones required for preparation of the reproductive tract for fertilization and subsequent supports pregnancy

Follicles are the functional units of the ovary: each follicle consist of an oocyte surrounded by one or more layers of somatic cells, the granulosa cells. Follicle development up to the ovulatory stage is a highly complex array of events.

The Primordial Germ Cells (PGCs) are the most primitive undifferentiated diploid sex cells, initially located outside the gonad. Oogenesis begins during the first month of gestation, between the 6th and 8th week, when PGCs migrate to the gonadal ridge from the yolk sac of the endoderm, the region that will form the future female gonad, and they differentiate into oogonia. These cells undergo a number of mitotic divisions and, by the end of the third month, are arranged un clusters surrounded by a layer of flat epithelial cells, the follicular cells. The majority of oogonia continue to divide by mitosis, but some of them arrest their cell division in prophase of meiosis I and form primary oocytes. During the next months, oogonia increase in number, and around 20 weeks of gestation they reach a total of 7 million, the maximum numbers. At this time, cell death begins, and many oogonia and primary oocytes become atretic. The surviving primary oocytes have entered prophase I and most of them are individually surrounded by a layer of flat epithelial cells; a primary oocyte together with the single layer of epithelial cells, called granulosa cells (GCs), forms a primordial follicle.

At puberty the number of primordial follicles is about 400,000, but only 400-450 of these will complete their maturation cycle(Baum et al., 2005), the total number of primordial follicles in the ovaries at any time is called the ovary reserve. The first event of follicologenesis is recruitment, a process by which an arrested primordial follicle is activated to resume development and enter the pool of growing follicles. As the primary oocyte begins to grow, surrounding follicular cells change from flat to cuboidal and proliferate to produce a stratified epithelium of GCs and the unit is called a primary follicle (Figure 3).

During primary follicle development, a molecular and biological internship between GCs and oocyte starts. Also, they form gap junctions with the oocyte cell membrane, or oolemma, that are important channels for the diffusion of ions, metabolites, and other signaling molecules such as cAMP and calcium. This communication between GCs and oocyte remains throughout follicologenesis and it is responsible for the synchronous expression of important activities.

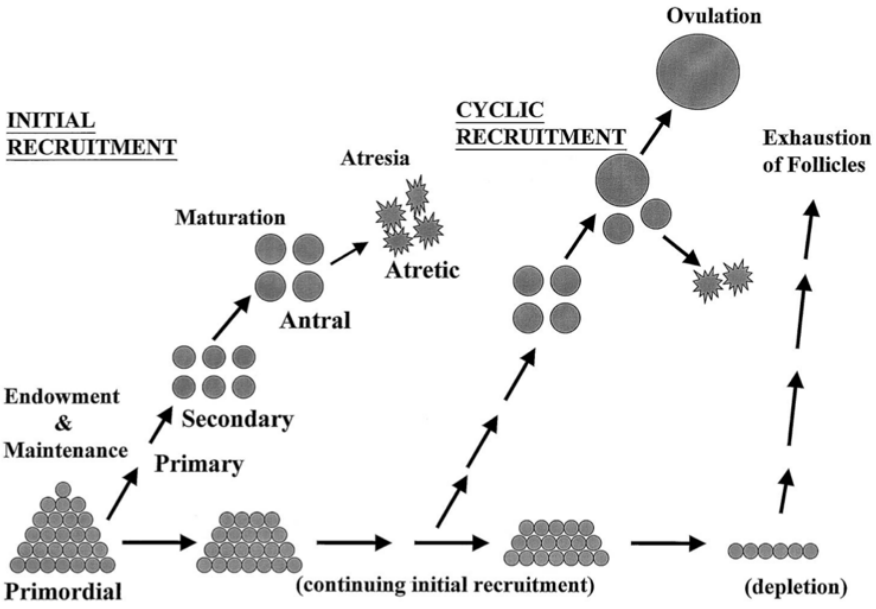

Figure 3. *Life history of ovarian follicles (McGee & Hsueh, 2000).*

The two most important developmental events that occur in the primary follicle are the expression of the FSH receptor by the cuboidal granulosa cells and the growth of the oocyte. GCs and the oocyte secrete a layer of glycoproteins on the surface of the oocyte, forming the ZP**.** The importance of the ZP is emphasized by the ZP-3 protein, the species-specific sperm-binding molecule, responsible for initiating the acrosome reaction in capacitated sperm.

At the puberty, the hypothalamus secretes the gonadotropin releasing hormone (GnRH) which stimulates the pituitary gland to secrete gonadotropins. These hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH) stimulate and control cyclic changes in the ovary.

At each ovarian cycle, 15 to 20 primary (preantral) stage follicles are stimulated to grow under the influence of FSH, without it these primary follicles die and become atretic, in normal conditions only one of these follicles reaches maturity and only one oocyte is released. FSH also stimulates maturation the monolayer of granulosa cells surrounding the oocyte, inducing their proliferation and forming the secondary follicle, characterized by somatic cells arranged in a multi-layered.

As secondary follicle development proceeds, two primary layers of theca appear: an inner layer of secretory cells, the *theca interna,* and an outer fibrous capsule, the *theca externa.* Theca development is also accompanied by the neoformation of numerous small blood vessels, presumably through angiogenesis. Consequently, blood now circulates around the follicle, bringing nutrients and gonadotropins to, and waste and secretory products from, the developing follicle. At the completion of the preantral phase of follicologenesis, a fully grown secondary

follicle contains five distinct but interacting structural units: a fully grown oocyte surrounded by a ZP approximately 9 layers of GCs, a basal lamina, a theca interna, theca externa and a capillary net in the theca tissue.

FSH stimulates the follicular cells to secrete a fluid, the follicular fluid (FF), that is released inside of this structure creating a cavity called "antrum", initially, the antrum is crescent shaped, but with time, it enlarges and form the tertiary follicle or Graafian follicle. GCs surrounding the oocyte remain intact and form the *cumulus oophorus;* the FF pushing the oocyte close to the wall of the follicle itself (Hennet & Combelles, 2012) (Figure 4).

At the antral stage most follicles undergo atresia; however, under optimal gonadotropin stimulation that occurs after puberty, a few of them are rescued (cyclic recruitment) to reach the preovulatory stage; the importance of FSH in supporting follicle growth after antrum formation and in preventing apoptosis has led to the concept that FSH is a survival factor for antral follicles.

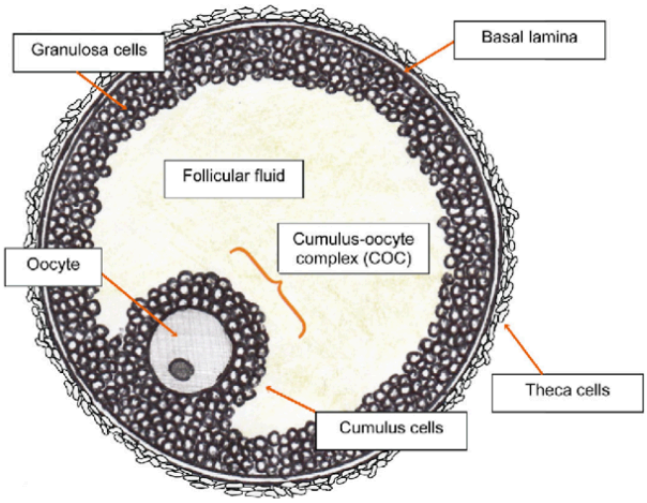

Figure 4. *Schematic representation of an antral follicle. Image from Hennet et al, 2012.*

FSH and LH also acts on the developing oocyte and they stimulate the completion of the first meiotic division, that result in formation of two daughter cells of unequal size, each with 23 homologous chromosomes: one cell, the secondary oocyte**,** and the *first polar body*.

The first polar body lies between the ZP and the cell membrane of the secondary oocyte in the perivitelline space.

The secondary oocyte undergoes the second division, which stops in metaphase II, instead the polar body degenerates. The oocyte, blocked in metaphase II, is expelled from the ovary, by a process called ovulation, and it can be fertilized (Pan & Li, 2019). Only with fertilization, the

second meiotic division is completed with the release of the second polar body, while in the absence of fertilization the oocyte degenerates.

Ovulation, is a process characterized the rupture of ovarian surface epithelium and the extrusion of the oocyte surrounded by the COC; the wall of the follicle becomes very thin and presents the stigma that is the point at which there will be the rupture of the follicle. The LH surge is necessary for the initiation of ovulation. In response to a peak of LH, the follicle breaks out and opens at the level of the stigma projecting outside the FF and the entire COC. At the time of ovulation, the follicle empties most of its content and the cavity is filled with a blood clot, turning into a yellow, expanded structure, which is called the corpus luteum If fertilization doesn't occur the corpus luteum remains active for twelve days and is called the corpus luteum albicans; if pregnancy begin, the corpus luteum increases in size and remains active for 3-4 months, and it becomes the corpus luteum gravidarum.

3.2 OOCYTE EVALUATION

The female gamete plays a crucial role in determining embryo competence and therefore in IVF results. Several factors can affect oocyte quality and a controlled ovarian stimulation (COS) protocol is one of the most important; in contrast to in vivo processes, the application of ovarian hormone stimulation protocols for IVF bypasses the complicated selection procedure that usually occurs during oocyte development and maturation of a single oocyte for ovulation, and allows for the maturation of many oocytes, often with compromised quality.

The quality assessment of oocyte is primarily based on its morphological features observed by light-microscope (Figure 5). Oocyte quality, and at the same time its development potential, is one of the essential factors determining the success of ART (Biase, 2017; Lazzaroni-Tealdi et al., 2015). Oocyte morphological assessment in the laboratory is first based on the aspect of the cumulus-corona cells. It is generally accepted that morphological evaluation of oocytes can be performed without damaging their initial developmental potential and subsequent embryonic development.(Lazzaroni-Tealdi et al., 2015).

One of the biggest problems during oocyte selection is the fact that even a normal looking oocyte can be a carrier of aneuploidy, therefore the research for new methods to simplify the selection oocytes with the highest development potential is in progress

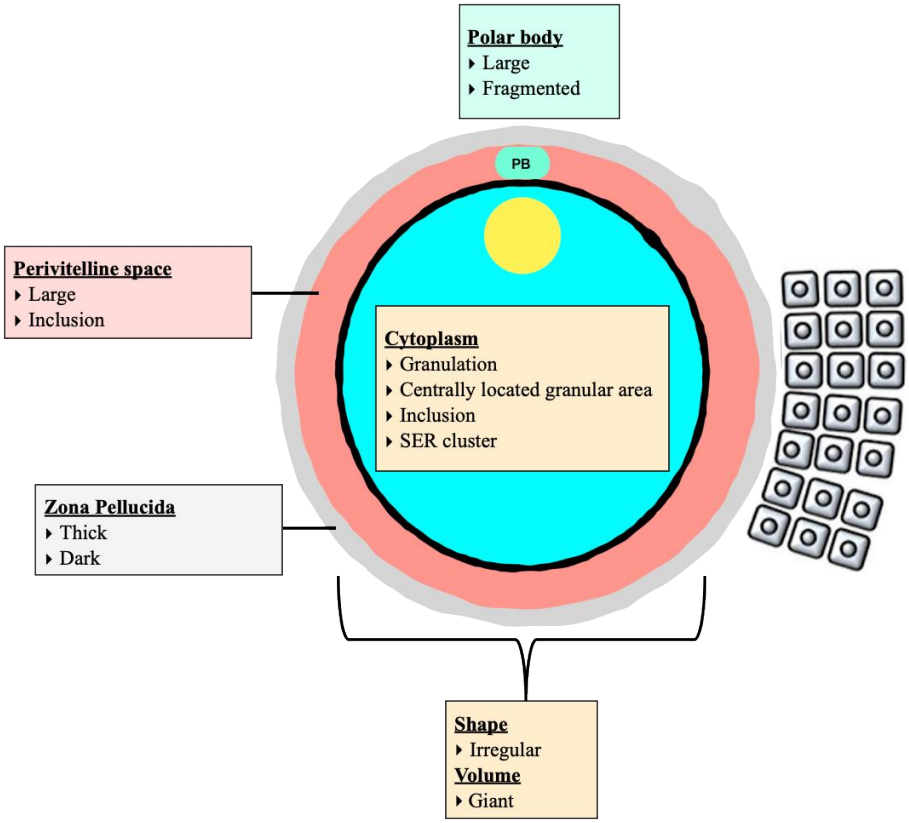

Figure 5. *Morphological properties used to assess oocyte competency.*

3.2.1 Cumulus-Oocyte Complex

MII oocytes and surrounding cumulus cells communicate with each other by using gap junctions for exchanging metabolites and regulatory molecules. The morphological features of a COC may help to determine the oocyte quality. During follicular antrum formation, GCs differentiate into mural GCs, lining the follicular wall, and CCs, surrounding the oocyte. The innermost layer of cumulus cells, immediately adjacent to the ZP, is called corona radiata. Cells of the corona radiata extend their cytoplasm toward the oocyte through the ZP.

In a mature oocyte the cumulus-corona mass should be and expanded layer, due to active secretion of hyaluronic acid. This extracellular matrix molecule interposes between the cumulus cells, separating them and conferring to the cumulus-corona mass a fluffy appearance.

COC abnormalities concern the type and degree of expansion of the cumulus mass and/or the pretense of blood clots.

Although many studies do not report a correlation between COCs morphology and nuclear maturity, fertilization rates and embryo cleavage, Lin and colleagues have proposed a grading system based on the morphology of the oocyte cytoplasm, cumulus mass, corona cells, and membrana GCs for oocytes prior to insemination by conventional IVF (Lin et al., 2003) as shown in Table 2.

Groups	OCCC morphology
Mature	Expanded cumulus Radiant corona Distinct zona pellucida, clear ooplasm Expanded well-aggregated membrane granulosa cells
Approximately mature	Expanded cumulus mass Slightly compact corona radiata Expanded, well-aggregated membrana granulosa cells
Immature	Dense compact cumulus if present Adherent compact layer of corona cells Ooplasm if visible with the presence of germinal vesicle Compact and nonaggregated membrana granulosa cells
Postmature	Expanded cumulus with clumps Radiant corona radiata, yet often clumped, irregular, or incomplete Visible zona, slightly granular or dark ooplasm Small and relatively nonaggregated membrana granulosa cells
Atretic	Rarely with associated cumulus mass Clumped and very irregular corona radiata if present Visible zona, dark and frequently misshapen ooplasm Membrana granulosa cells with very small clumps of cells

Table 2 Oocyte–corona–cumulus complex evaluation scheme (Adapted from Lin et al., 2003)

The authors reported higher fertilization rates for oocytes belonging to the mature group compared with those belonging to the other groups, probably the presence of cumulus-corona cells may help embryonic metabolism by stimulating gene expression (McKenzie et al., 2004) or reducing oxidative stress (Fatehi et al., 2005).

However, there is little evidence to support the role of COC morphological analysis in predicting oocyte maturity and competence (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). Recently, it has been proposed a simpler score based only on cumulus cell expansion (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011), in fact there is a higher probability of obtaining a better quality mature oocyte in a normally expanded cumulus than in an unexpanded one (Canipari R, Camaioni A, Scarchilli L et al., 2004).

It was noted that the presence of blood clots in CC may adversely affect the rates of fertilization and cleavage of early embryos derived from corresponding oocytes (Daya et al., 1990), and decreases these oocytes quality and blastocyst formation rate (Ebner et al., 2008).
More interesting approaches have been developed in order to better understand oocyte quality on the basis of cumulus cells and GCs RNA/protein content and metabolite production but this

approach us currently under investigation and more studies are needed (Huang & Wells, 2010; Uyar et al., 2013).

3.2.2 Oocyte nuclear maturity evaluation

Direct observation of oocyte morphology, including the extracytoplasmic components, is possible only after the denudation of its cumulus and corona layers. The use of hyaluronidase enzyme and mechanical pipetting facilitates the breaking down of the cumulus–corona extracellular matrix. This method is normally used when insemination by ICSI is going to be performed.

Currently, oocyte nuclear maturity is determined by the presence of an extruded IPB in the PVS and by the absence of a GV. Approximately 85% of the denuded oocytes display the IPB and are classified as MII. In about 10% a GV is present in the oocyte cytoplasm; approximately 5% of the oocytes are in the metaphase of the first meiotic division (MI) with no visible GV and IPB (Revelli et al., 2003).

3.2.3 MII oocyte morphological evaluation

Morphological abnormalities of a metaphase II oocyte after denudation of the corona-cumulus layer may be examined under two subgroups:

- Extracytoplasmic abnormalities: shape and size, ZP (dark or thick), and perivitelline space (large or granularity)
- Cytoplasm abnormalities: ooplasm (granulation or variations in color),

A “normal” human MII oocyte should have a round, clear ZP, a small perivitelline space containing a single, non-fragmented first polar body, and a pale, moderately granular cytoplasm with no inclusions (Figure 6); however, the majority of the oocytes retrieved after ovarian stimulation exhibit one or more morphological abnormalities involving the cytoplasm aspect and/or the extracytoplasmic structures (Balaban et al., 1998; De Sutter et al., 1996; Ebner et al., 2006)

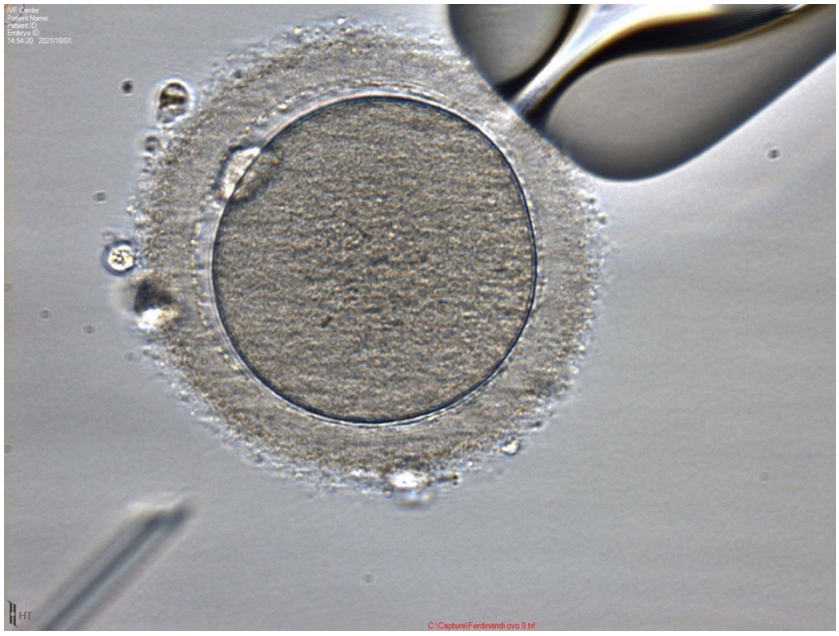

Figure 6. *MII oocyte with normal morphology (400x magnification)*

Extracitoplasmatic features

- *Oocyte size and shape:* good-quality mature human oocytes have a round and clear zone pellucida. Oocytes with extremely abnormal shape are regularly reported (Figure 7). The diameter of MII oocytes vary substantially but it is not related to fertilization or developmental quality of embryos (Romão et al., 2010), the situation is different with giant oocytes (Balakier et al., 2002) in fact this type of oocyte has about twice the volume of a normal oocyte (about 200 mm) and is tetraploid before meiosis due to either nuclear division but no cytoplasmic division into one oogonium or from the cytoplasmic fusion of two oogonia (Austin, 1960).

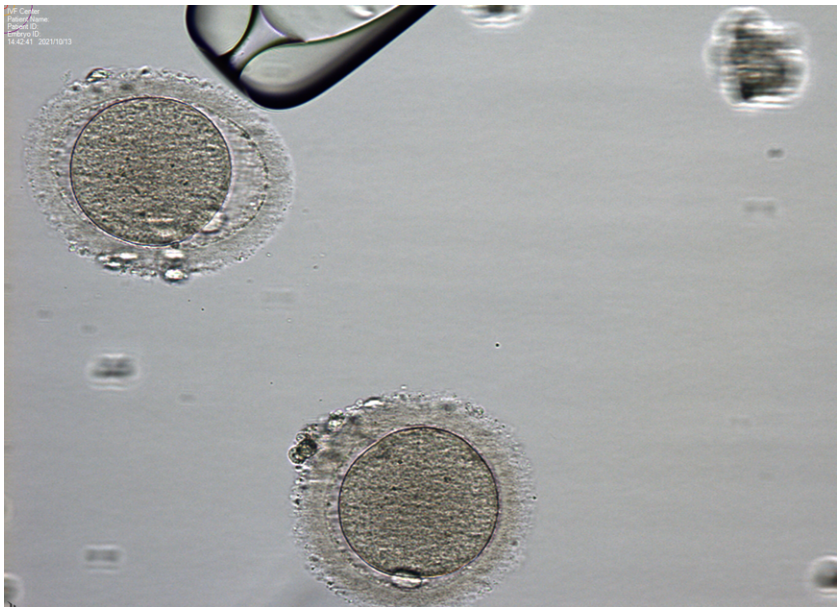

Figure 7. *MII oocyte with abnormal shape (200x magnification)*

- *Zona pellucida:* oocytes are surrounded by ZP that is generated both by oocytes and enclosing GC in the primary follicles, so anomalies may result from perturbations of follicologenesis and reflect an altered oocyte quality. The ZP may show several morphological abnormalities including changes in thickness or increased density associated with reduced translucency, a feature known as "dark ZP" (Figure 8). The complete absence of the ZP is extremely rare (Stanger et al., 2001), but more subtle changes in structure are more frequently observed (Rienzi et al., 2012).

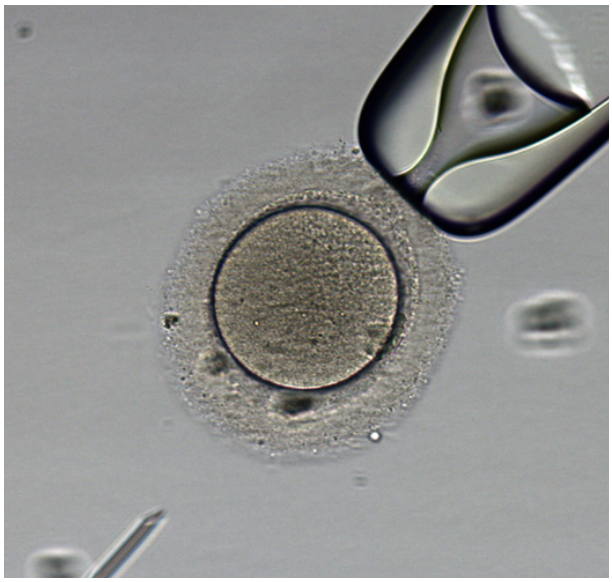

Figure 8. *MII oocyte with dark ZP (200x magnification).*

- *Perivitelline space:* the perivitelline space is the volume bounded by the PVS and not occupied by the oocyte. Generally, normal MII oocytes have a small PVS that includes individual polar bodies (PB) and no granulated dispersed material So the two morphological features that are usually evaluated are: size (enlarged or not) and content (presence of granulation) (Figure 9).

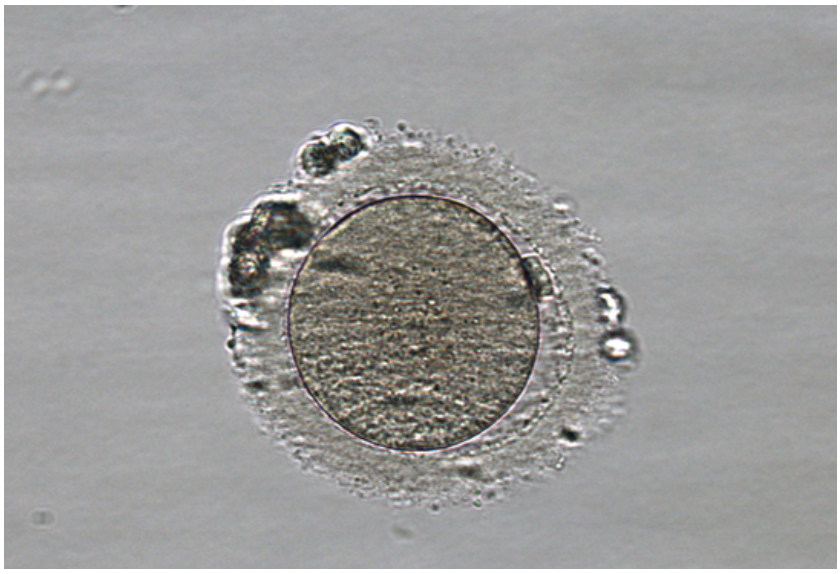

Figure 9. *MII oocyte with granulation in PVS (200x magnification).*

- *First polar-body:* PBI is extruded to eliminate one of the two copies of each homologous chromosome at the first meiotic division; therefore, correct extrusion of the PBI indicates successful meiotic maturation of the oocytes. For this reason, the morphology of the polar body could represent a marker of quality and even aging status of the oocytes because in vivo aged MII oocytes had a degenerated PB due to over maturity (Eichenlaub-Ritter et al., 1995) (Figure 10).

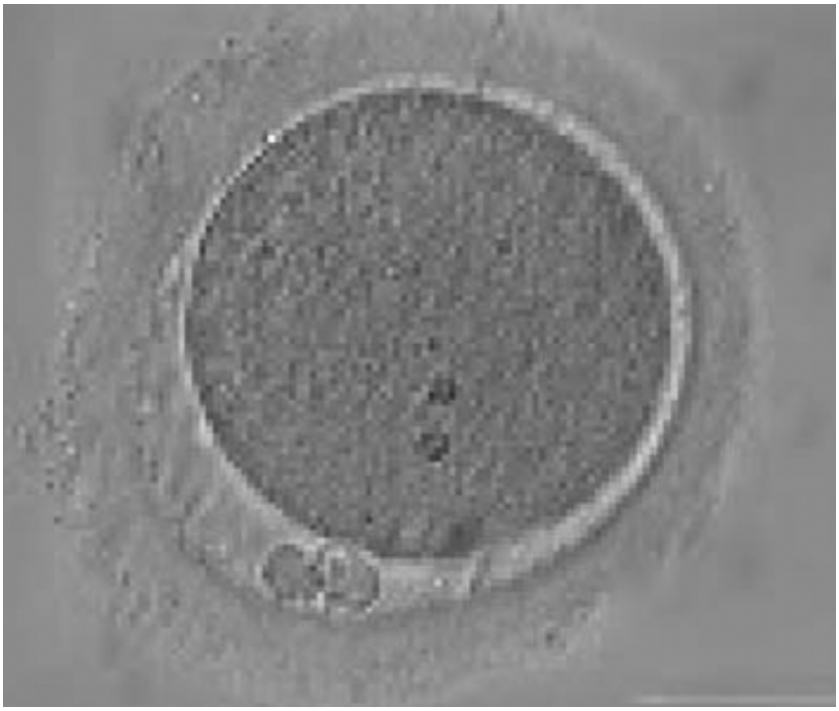

Figure 10. *MII oocyte with fragmented PBI (200x magnification).*

Cytoplasmic features

It is generally reported that the cytoplasmic texture is a very important characteristic for oocyte selection (De Sutter et al., 1996; Rienzi et al., 2011). Cytoplasmic changes such as vacuolization and various types of incorporations including refractile bodies, granulation, inclusion, discoloration, and clustering of the smooth endoplasmic reticulum (SER) in the oocytes were evaluated for the purpose of predicting their potential effects on developmental competence (Ebner et al., 2001)**.** Based on current evidence, slightly heterogeneous cytoplasm may only represent normal variability among retrieved oocytes rather than being an abnormality of developmental significance (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011) but severe dysmorphism of the cytoplasmic texture impairs the developmental and implantation potential of the embryo (Balaban & Urman, 2006). One of the most important severe cytoplasmic abnormalities of MII oocytes is the appearance of smooth endoplasmic reticulum clusters (SER); aggregate of SER appear as a flat disks in the oocyte cytoplasm corresponding to large tubular SER cluster surrounded by mitochondria(Sá et al., 2011). Evidence-based data clearly demonstrates that the embryos derived from oocytes with SER discs are associated with the risk of significantly abnormal outcomes (Balaban & Urman, 2006) and it is recommended that oocytes with this feature should not be used for injection.

Cytoplasmic alteration, such as granularity (Figure 11,12) and presence of inclusions (Figure 13) may be a sign of cytoplasmic incompetence. The potential effects of cytoplasmic inclusion on developmental progress were analyzed by Xia (Xia, 1997) who reported that the presence of inclusions was highly correlated with a lower fertilization rate and poor embryonic quality and that cytoplasmic inclusion in oocytes was at higher levels in patients older than 35 years than in patients younger than 35 years. This study also suggests that cytoplasmic inclusions might exhibit their adverse effect(s) at the later terms of prenatal development. However in other studies, no correlation was detected between cytoplasmic inclusions and the development-related parameters such as implantation, fertilization, and embryo quality rates (Balaban et al., 1998; De Sutter et al., 1996).

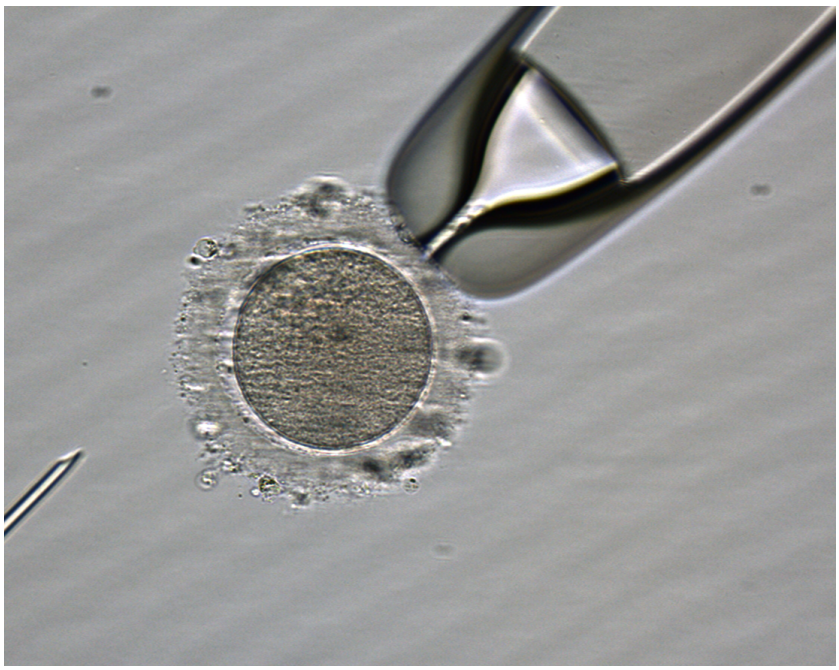

Figure 11. *MII oocyte with granular cytoplasm (200x magnification).*

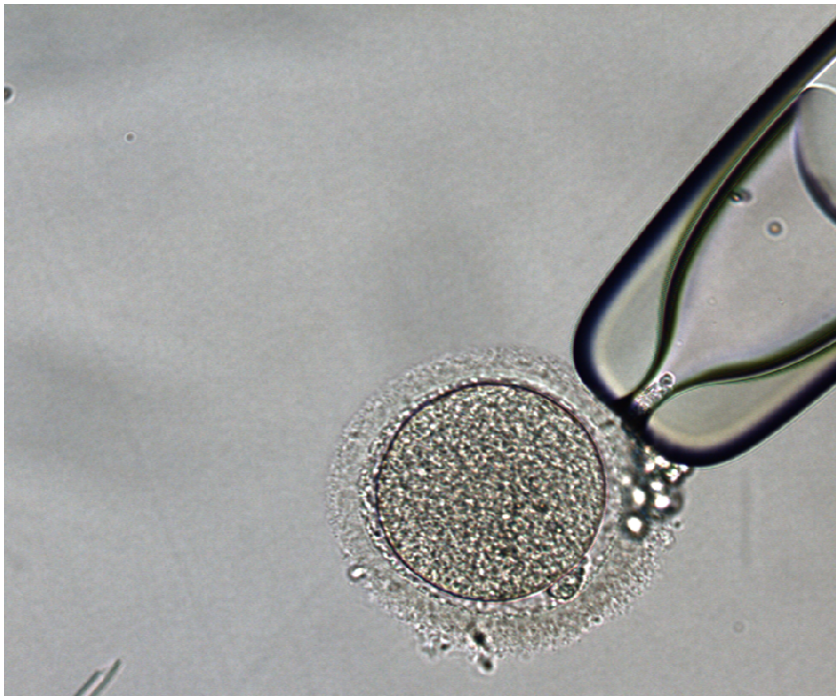

Figure 12. *MII oocyte with a high degree of cytoplasmic granularity (200x magnification).*

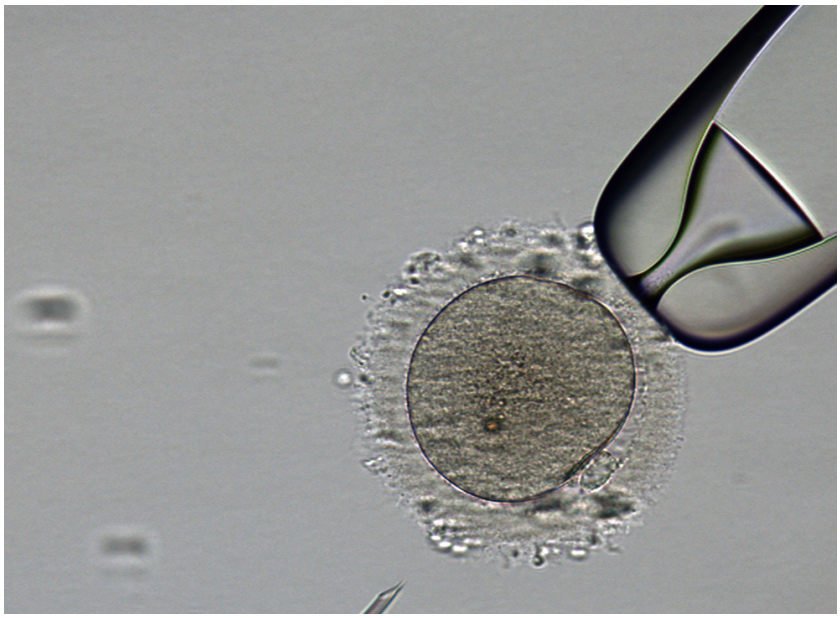

Figure 13. *MII oocyte with inclusion in cytoplasm (200x magnification).*

4 ARTIFICIAL INTELLIGENCE (AI) IN THE IVF LABORATORY

The field of reproductive medicine is constantly evolving, with new techniques continually being introduced to implement and improve clinical outcomes.

New technologies, such as better cameras and data capturing systems, are rapidly becoming an integrated part of the fertility clinic and result in a vast amount of stored data, including patient data, oocyte images, embryo time-lapse videos. The introduction of mathematical AI techniques into ART could lead to another revolution. In recent years, AI has proved to be a valuable tool in medicine by analyzing large amount of data; several algorithms have been applied to the classification or prediction of reproductive medical data by applying classical statistical methods such as logistic regression, or in other cases using different AI techniques.

To explore the potential application of AI, we have to first define it, and how it differs from machine learning and deep learning technologies (Figure 14).

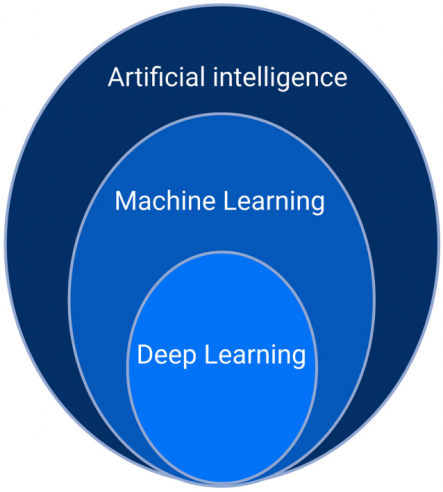

Figure 14. *Hierachy of information processing theories in the computer science field.*

AI is a term that describes machines that aim to mimic human or animal cognitive capacity.
Machine learning is a subset of the broader AI technology that learns its processing from data without explicit programming. It can predict outcomes for tasks that comprise many parameters and that would be too time-consuming (or impossible) for a person to perform.
Deep learning is a further subset of machine learning. It utilizes artificial neural networks (ANN) which mimic the architecture of neurons in the brain.
The introduction of AI could be useful at different stages of the IVF procedure such as gamete and embryo selection as well as in the development of a treatment regimen.
Focusing on oocyte selection, the use of AI techniques suggests an improvement in the results, or at least an automation of the process. In all cases, the main objective is to develop systems that are able to automatically support the decision of which oocytes to inseminate. In addition, the systems can improve the success rate of infertility treatments in the clinics, helping to make correct decisions in complex cases.
Current clinical assessment of gamete health is focused on identifying early markers of quality. This includes visualization of gametes either through direct inspection or via static images or time-lapse videos.
The quality of female gametes is associated with oocyte morphology, and cytoplasmic characteristics. However, the selection is subject to a high degree of variation between operators; AI can be crucial in this aspect, because it removes the subjectivity of human assessment from the decision-making process, and objectively rank gametes based on quality. Some early attempts have already been made with AI methods to evaluate human oocytes and predict normal fertilization, assess embryo development to the blastocyst stage, and even analyze implantation potential using static oocyte images, such capacity appears limited based

on the current literature (Manna et al. 2013), but these initial results need to be confirmed by larger studies

4.1 Artificial neural networks

The neurons of the human brain form an intricate and interconnected network. They are highly specialized cells in the reception and conduction of electrical impulses, and being able to transmit information, as electric pulses, to nearby and connected neurons. The learning process occurs by activating these cells, trough the propagation of action potential. This is a poorly understood and complex biological process.

The ANN's attempt to simplify and mimic this brain behavior.

The AI techniques most used by now in assisted reproduction are MLP and CNN.

Multilayer Perceptron

The main type of ANN is the Multilayer Perceptron (MLP). The simplest architecture of the MLP consists of many small elementary units, the nodes, distributed in three different layers: input layer, output layer and one hidden layer (Figure 15). Except for the input nodes, each node is a neuron that uses a nonlinear activation function; each node in one layer is connected to every node in the following layer. The input layer receives the input signal to be processed while the output layer produces the result of network processing.

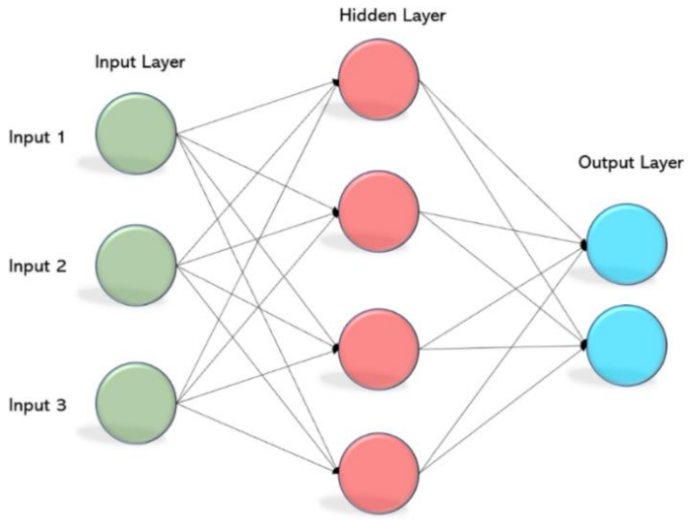

Figure 15. *Representation of the architecture of MLP.*

In the field of assisted reproduction, many problems can be solved using the ANN. This technique has a wide range of applications because it can be used to solve problems like classification, prediction or sample selection. For example, in ART the classification and

selection of oocytes, sperm and embryos that are routinely conducted by embryologists can be facilitated by AI-based tools.

The universal approximation theorem states that an MLP can approximate any type of function with any precision, even with a single hidden layer, as long as it is sufficiently "wide" (Hornik, 1991). The lack of success in applying the model results from inadequate learning, an insufficient number of neurons, or the lack of a deterministic relationship between inputs and outputs; for example the feature selection is an important pre-processing steps, which may profoundly impact the performance of the AI model; this process consists of determining the relevant data and removing the irrelevant and noisy data that offer no useful information (Kumar & Minz, 2014).

There are many studies in the literature that use the MLP architecture to classify different types of ART data: some of these attempted to determine semen quality through the use of information on the lifestyle and environment of the patients [18-20]; others papers have focused on other factors involved in the success of IVF treatment such as women's physiology parameters (FSH, AMH, PG) the number of oocytes retrieved and the sperm quality parameters. Furthermore, with the rise of time-lapse technology a new perspective to the development of the embryo was revealed and a new way to analyze its competence has also emerged since the time-lapse monitoring generates a huge amount of data that can been analyzed by the AI techniques.

Convolutional neural network (CNN)

Deep learning is a modern extension of the classical neural network technique, capable of building neural networks with a larger number of layers. That is possible due to the rapid development of modern computing.

The name "convolutional neural network" indicates that the network employs a mathematical operation called convolution, which is a specialized kind of linear operation. Convolutional networks are simply neural networks that use convolution in place of general matrix multiplication in at least one of their layers [45]

The convolution layer is the core of the CNN, where the main portion of the network computation occurs. An image consists of a matrix of elements; the core of the CNN is a smaller matrix that has a specific task such as detecting lines or edges. The CNNs can be used for tasks such as object detection and semantic segmentation; they are used primarily for images, such as in the process of classification and clustering.

Khan et al. applied the CNN to time-lapse images to count the numbers of cells in developing embryos.

5 Research study purpose

The success of ART depends on each individual step in the procedure. An adequate evaluation of the oocyte morphology can represent a measure complementary to embryonic evaluation to achieve embryo selection and maximize efficiency. Routine embryo assessment is well codified by internationally recognized criteria for semi-quantitative characterization. On the contrary, the assessment of the oocyte morphology is still performed more arbitrarily. The question of oocyte dysmorphisms is highly prevalent, following cumulus cell removal, approximately 60–70% of oocytes show abnormal morphological characteristics. Since dysmorphisms may be associated with cytoskeletal and other cellular defects, indiscriminate use of abnormal oocytes might imply the risk to generate developmentally incompetent embryos. This could affect pregnancy rates or result in further negative consequences, such as miscarriages or neonatal malformations.

Furthermore, can an artificial intelligence (AI)- based model predict oocyte quality and potential using images captured by optical light microscopy? This work concentrates the efforts on the possible prediction of the quality of oocytes in order to improve the performance of assisted reproduction technology, starting from their images. In most cases, embryologists select them by visual examination and their evaluation is totally subjective. The artificial intelligence system proposed in this work is based on a ResNet that has been trained both to identify oocytes and two cytoplasmic anomalies. We also tried to develop an algorithm capable of predicting the outcome of fertilization based on the couple's anamnestic data.

6 MATERIALS AND METHODS

6.1 Patients Selection

The study included 212 consenting patients treated by ICSI at A.G.I. Medica (Siena) from September 2018 to September 2021.

6.2 Assisted Reproduction Techniques

In all cycles controlled ovarian stimulation was performed using recombinant FSH (rFSH; GonalF Merck Serono, Italy and Puregon, MSD, Italy) or human menopausal gonadotropin (hMG, Meropur, Ferring, Italy), the choice of using one or another depends on the patient's anamnesis, considering in particular previous IVF treatments and the response to them in terms of oocytes retrieved and fertilization rate. The starting dose is based on factors like antral follicle count, AMH levels, basal FSH (on day 2 or 3 of a normal menstrual cycle), patient's age, with the aid of nomogram of Antonio La Marca (Papaleo et al., 2016). Ovarian stimulation normally lasts from 12 to 16 days and it normally starts on day 2 or day 3 of the menstrual cycle, induced with estroprogestin pill or progesterone (norethisterone acetate 10 mg) when pituitary desensitization was achieved as defined by the absence of ovarian follicles >10 mm and endometrial thickness < 4 mm on transvaginal ultrasound examination. An ultrasound follicular monitoring and a serum dosage of estradiol and progesterone were done every 2 or 3 days and gonadotrophin doses were adjusted according to the ovarian response. A GnRH antagonist, like cetrorelix or ganirelix (Cetrotide, Merck Serono, Italy, Orgalutran, MSD, Italy) was introduced with an estradiol dosage > 300 ng/ml and at least one follicle with medium diameter of mm 14. When serum estradiol concentration exceeded 1000 pg/ml and at least three follicles reached 18 mm in diameter, 250 mcg of rhCG were administrated.

Oocyte retrieval was performed 34-38 hours after hCG administration. Follicles were aspirated with a negative pressure of 115-120 mm Hg with a single lumen 17-gauge oocyte pick-up needle under transvaginal ultrasound guidance. No flushing of the aspirated follicles was performed.

Semen sample was examined according to the WHO (2010) (Table 3) and the patients were divided into two groups: one group with normal semen parameters (n = 77) and the other group with semen parameters below the 5th percentile, which is considered to be the lower limit of normal (n = 56).

PARAMETER	CENTILE				
	5	25	50	75	95
Semen volume	1,5	2,7	3,7	4,8	6,8
Total sperm number (10^6 per ejaculate)	15	41	73	116	213
Sperm concentration (10^6 per ml)	39	142	255	422	802
Total motility %	40	53	61	69	78
Progressive motility %	32	47	55	62	72
Normal forms %	4	9	15	24,5	44
Vitality %	58	72	79	84	91

Table 3. *Reference values for human semen characteristics (World Health Organization, 2010)*

Oocytes were denuded from the cumulus oophorus by a brief exposure to hyaluronidase solution (Hyaluronidase Irvine Scientific®), followed by mechanical removal of the corona radiata with the use appropriate pipette with progressively smaller caliber tips (170-140 μm). At this point it is necessary to evaluate the meiotic status of the oocytes using the stereomicroscope:

- MII oocytes: they have extruded the first polar cell and are therefore mature and ready for injection.
- MI oocytes: have not yet extruded the first polar body, are not suitable to be inseminated;
- Germinal vesicle (VG): they have not extruded the first polar body and have still well still visible in the center of the oolemma the germinal vesicle; they are not suitable to be inseminated.

MII oocytes were separated from the immature oocytes and cultured until the insemination.

Oocytes was subjected to ICSI using previously described technique by Palermo et al. (1992).

After about 16-18 hours, fertilization was evaluated by observing the presence and correct number of pronuclei in the zygote (2 pronuclei). Only those oocytes that showed two pronuclei and two polar bodies were considered normally fertilized. Abnormally fertilization was excluded.

Cleaving embryos were evaluated on day 3 after ICSI (72 h) taking into account the number of blastomeres, that represents the cleaving status of the embryo. Additionally, the embryos are graded as following: (Veeck 1999)

- Grade 1 - embryo with blastomeres of equal size, no cytoplasmic fragments;
- Grade 2 - embryo with blastomeres of equal size, minor cytoplasmic fragments or blebs;
- Grade 3 - embryo with blastomeres of distinctly unequal size; none or few cytoplasmic fragments;
- Grade 4 - embryo with blastomeres of equal or unequal size; significant cytoplasmic fragmentation;

Embryos to be transferred on day 3 are selected based on this analysis

The luteal phase was supported by means of natural progesterone in oil, 50 mg/day IM (Pleyris) starting on the day after the HCG injection.

Pregnancy was confirmed by a serial rise in serum HCG concentration on two consecutive occasion 15 days after embryo replacement.

6.3 Oocyte Morphology assessment

The morphology of the inseminated oocytes was evaluated at the time of ICSI (from 2-4 hours after retrieval) under inverted microscope at x400 magnification by a single operator and annotated using the MedITEX IVF Software (Figure16).
A single defect per category (Table 4) was noted and a score of 1 was assigned for each category assessed as normal; the score ranges from 0 to 5 for those oocytes that have normal morphology.

Figure 16. *Detail of oocyte classification system in MedITEX IVF software.*

OOPLASM	GRANULAR
	CENTRALLY LOCATED GRANULAR AREA
	INCLUSION
	SER CLUSTERS
	NORMAL
SHAPE	IRREGULAR
	GIANT
	NORMAL
PERIVITELLINE SPACE	LARGE
	INCLUSION
	NORMAL
I POLAR BODY	LARGE
	FRAGMENTED
	NORMAL
ZONA PELLUCIDA	THICK
	DARK
	NORMAL

Table 4. *Morphological features used to assess oocyte score in MedITEX IVF software.*

6.4 Statistical analysis

Categoric variables describing oocyte features are presented as absolute and percentage frequency with respect to both total oocytes and total patients involved.

Bivariate analyses between morphologic oocyte features and ICSI outcome (fertilization, embryo quality) were performed using the Pearson chi-squared test.

Statistical significance was defined as $P < 0.5$.

6.5 Artificial Intelligence

In collaboration with the Department of Information Engineering and Mathematic Sciences, we have undertaken a research project based on the application of integrated AI systems to assisted reproduction.

The project was carried out on two fronts, necessarily synergistic. On the one hand the acquisition and processing of clinical data; on the other, the application of systems of

application based on artificial intelligence techniques able to support the embryologists during the use of the ICSI technique, intervening in the most critical phases of the process.

The case histories have been recruited at the center AGI Medica, with appropriate anonymization of data so as not to be able to trace the names of patients.

We collected and processed a range of data inherent in the descriptive characteristics of the problem (e.g., the couple's medical history). For each couple that turned to the center, data were collected such as: infertility factor, BMI of the patient, quality of the sperm, the characteristics of the oocytes and the outcome of fertilization.

Once completed the collection and processing of data, the study project has been articulated in four fundamental phases, based on the application of artificial neural networks

1) Training of a network for the individuation of an oocyte: it has been trained a ResNet network (extremely deep network without considerable increase of the complexities) with the objective of identifying the oocytes visible within the images made available by the clinic.

2) Analysis of oocyte cytoplasmic abnormalities: two different algorithms have been created, the first identifying the granularity of the cytoplasm by analyzing the textures, and the second that identifies the presence of cytoplasmic inclusions.

3) Enrichment of the dataset: the dataset available was initially composed of only 97 oocyte images. In order to train the classifier described above, it was found necessary to create additional images of oocytes.

4) Creation of an additional classifier for the analysis of descriptive tabular data of the couple with the aim of predicting the outcome of the meeting of the two gametes.

6.5.1 Oocyte identification

The first phase of the study consisted in the identification, through techniques of "object detection", of the oocytes within the images. For this purpose, has been a specific neural architecture was trained. The initial dataset consisted of ninety-seven images of varying variable size (1360X1024, 1211X915, 1175X885), containing one or more oocytes. To improve the performance of the system, the original dataset was enlarged through the creation of one thousand synthetic images of oocytes with presence of cytoplasmic inclusions, and as many of oocytes without any abnormality.

Overall, for the initial phase (oocyte identification), a dataset of 117 images was used: 97 used for the training set and 20 for the test set.

On this dataset, we proceeded to the identification and highlighting (bounding box) (Figure 17), through techniques of object detection techniques (a set of activities in the field of computer vision designed to detect objects in digital images), of the oocytes within the images.

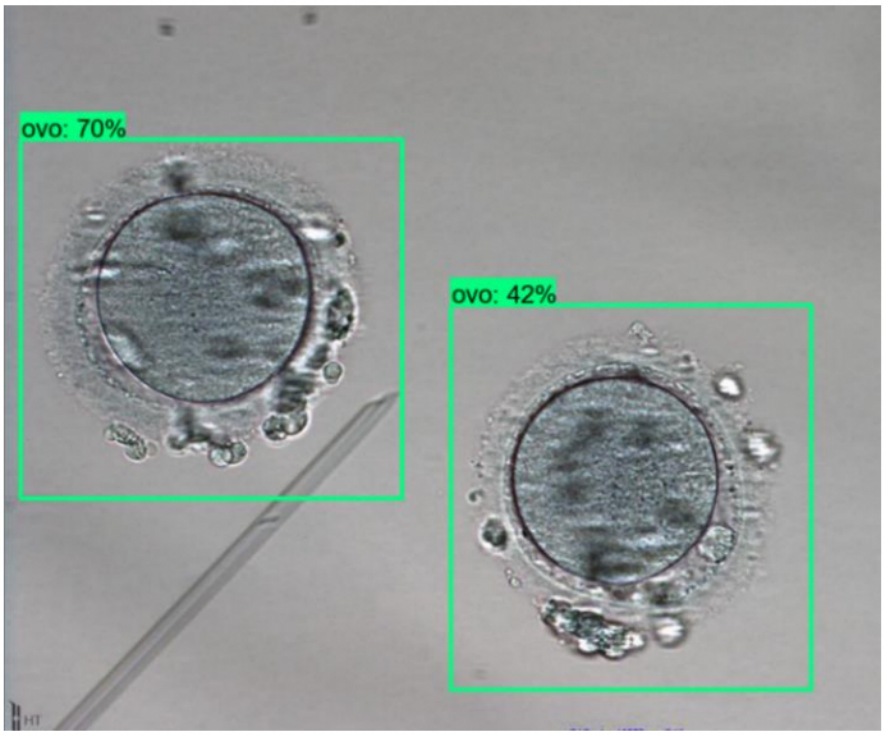

Figure 17. *Example of object detection on real images.*

6.5.2 Identification of cytoplasmic abnormalities

Once the oocytes were successfully identified, the attempt was undertaken to use networks and architectures of AI for the identification of cytoplasmic abnormalities of the oocytes themselves. Very briefly, once the oocyte is identified in the image, the oocyte is isolated and post-processed so that cytoplasmic abnormalities can be identified. Specifically, the application cuts the image around the "bounding box" previously identified and, the extracted part, is resized to a size to a size of 256x256 pixels. The next step is to apply a circular mask over the resized image, in order to isolate the pixels that identify the cytoplasm. The experiments that have been conducted have aimed the identification of two particular cytoplasmic abnormalities: the granularity of the cytoplasm and the presence of cytoplasmic inclusions.

In particular, for the granularity of the cytoplasm, which appears visually as an abnormal roughness, the application analyzed the variance of pixels in the image masked. The oocyte was labeled by the application as granular if the variance exceeds the threshold of 1400, otherwise, the oocyte is labeled as non-granular. The result of the classification performed by the application was reviewed together by the center's team of expert biologists. At the end of the process, we have identified cases where the application correctly identified granular cytoplasm, which in contrast, in the tabular data were labeled as non-granular.

For the identification of cytoplasmic abnormalities that appear as spots that are irregular black spots of varying sizes within the cytoplasm (Figure 18), a neural network was created neural network that implements an oocyte classification based on the presence of the inclusions in the cytoplasm. Due to the scarcity of available images, in order to create a sufficiently large dataset to train the classifier, it has been the need to use systems for the creation of synthetic images through the use of particular architectures.

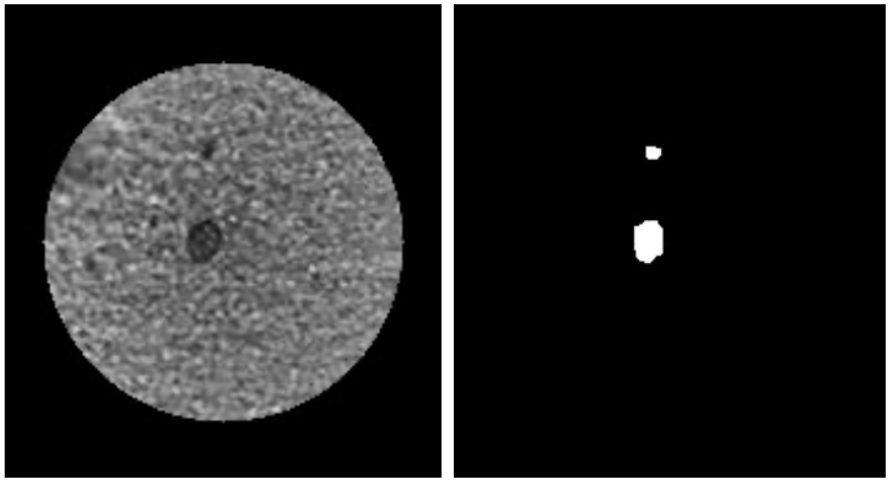

Figure 18. *On the left, the image of a cytoplasm with the presence of cytoplasmic inclusions, on the right, the corresponding binary mask.*

By means of such systems have been produced 1000 synthetic images of oocytes with the presence of cytoplasmic inclusions (Figure 19). We have, together with a team of expert biologists of the center, qualitatively evaluated the images produced by the network, finding no defects or substantial differences from the real images. We proceeded to classify images containing cytoplasmic inclusions through a convolutional network, a deep architecture, inspired by the biological model biological model of connectivity of neurons in the human brain and, in particular, of the visual cortex, which shows excellent performance in image processing.

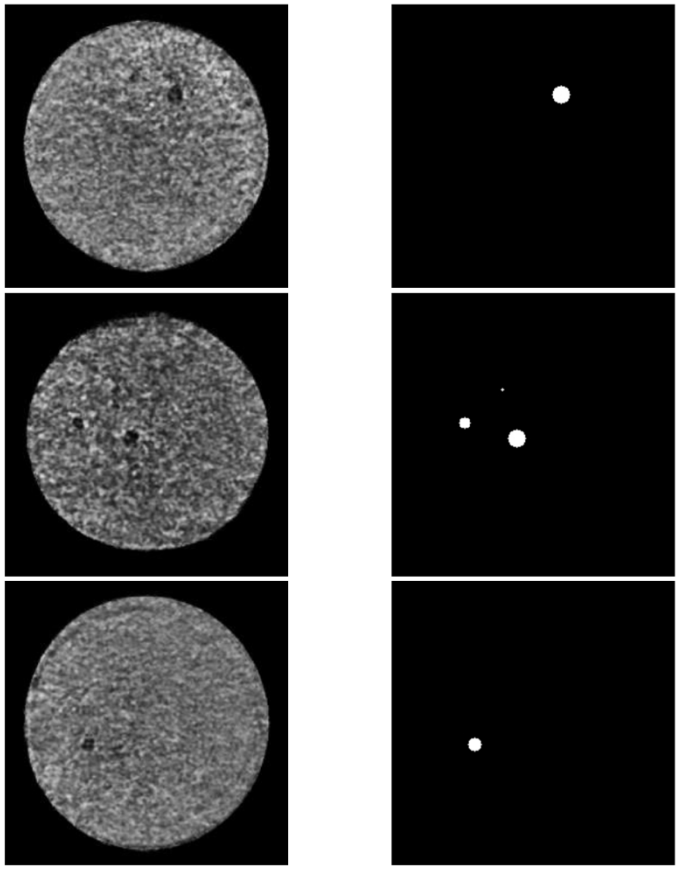

Figure 19. *Samples of images generated using PIX2PIX and their masks*

6.5.3 Prediction of the outcome of fertilization

The last part of the study, was that relating to the prediction of the outcome of fertilization from anamnestic data. For the purposes of this part of the study project, tabular data (oocyte and seminal fluid history, male factor) collected in advance in an Excel spreadsheet containing 1255 records were used.

The tabular data used for the prediction of fertilization were preprocessed in order to optimize the training process of the classifier. After a process of transformation of the reference variables of the categorical type, in "dummy" variables (i.e., variables of numerical type), we proceeded to insert these variables in the classifier. After a process of elimination of repeated data or records lacking in information and further processes of adjustment, it was obtained a balanced dataset containing the same number of examples belonging to class 1 (successful fertilization) and class 0 (unsuccessful fertilization), consisting of 382 samples. MLP architecture was used for this application, which generates a non-linear function model that allows the prediction of the output data based on the input data.

7 RESULTS AND DISCUSSION

The mean age of the 212 patients included in the present study was 36,2 ± 4,2. During the study period, 1508 CCO complexes were retrieved in 217 cycles, out of which 1254 were at MII stage (83,1%). The number of eggs collected per retrieval was 6,9 ± 4,6. Other patient characteristics are summarized in table 5.

	n°	Mean or %	SD	Min	Max
Female age	212	36,2	4,2	24	45
Female BMI	212	23	4,7	16,8	39,9
AMH	212	2,7	2,6	0,18	12,43
Diagnosis*					
Endometriosis	18	8,4%			
Endocrine disorders	11	5,1%			
PCO	4	1,9%			
DOR	71	33,5%			
Advanced maternal age	24	11,3%			
Genetic disorders	5	2,3%			
Tubal factor	25	11,7%			
Recurrent pregnancy loss	10	4,7%			
Male factor	36	16,9%			
Unexplained	32	15%			

Table 5. *Patient characteristics. (*Patients may have more than one diagnosis)*

7.1 Incidence of the different Oocyte Morphologic Features

Out of the 1254 oocytes evaluated, 80.3% (1008 oocytes) had at least one morphological defect. The most frequent abnormalities are those related to the ooplasm, were observed in 708 oocytes, among them the most represented defect is the presence of inclusion in the cytoplasm as can be seen in the table. Then we find the abnormalities of the perivitelline space, found in 571 oocytes (45,5%). Abnormal IPB and ZP were observed in 413 (32,9%) and 189 (15%) of the analyzed oocytes, respectively. Abnormal shape was relatively rare.

In 95,8% of the patients involved in the present study, at least one oocyte was recorded as morphologically “abnormal”.

FEATURES		
OOPLASM 708 OOCYTES (56,4%)	**GRANULAR**	223 OOCYTES (17,7%)
	CENTRALLY LOCATED GRANULAR AREA	42 OOCYTES (3,3%)
	INCLUSION	381 OOCYTES (30,3%)
	SER CLUSTERS	62 OOCYTES (4,9%)
SHAPE 89 OOCYTES (7%)	**IRREGULAR**	79 OOCYTES (6,2%)
	GIANT	10 OOCYTES (0,79%)
PERIVITELLINE SPACE 571 OOCYTES (45,5%)	**LARGE**	25 OOCYTES (1,9%)
	INCLUSION	546 OOCYTES (43,5%)
I POLAR BODY 413 OOCYTES (32,9%)	**LARGE**	40 OOCYTES (3,1%)
	FRAGMENTED	373 OOCYTES (29,7%)
ZONA PELLUCIDA 189 OOCYTES (15%)	**THICK**	171 OOCYTES (13,6%)
	DARK	18 OOCYTES (1,4%)

Table 6. *Incidence of morphological defects in oocytes.*

7.2 Relationship between Oocyte Morphology Score and Fertilization Rate

The fertilization rate obtained for oocytes with no abnormalities was 71,9%. As can be seen from Table 7, oocyte morphology was not related to fertilization rate.

	TOTAL				
SCORE	**N° OOCYTES**	**2PN**	**NO PN**	**% FERT**	**P value**
0	1	0	1	0	NS
1	35	25	10	71,4	NS
2	226	182	44	80,5	NS
3	384	291	93	75,7	NS
4	362	268	94	74	NS
5	246	177	69	71,9	NS
TOTAL	1254	943	311	75,1	NS

Table 7. *Correlation between oocyte score and fertilization rate.*

The oocytes that showed a better degree of fertilization were those with a score of 2, thus presenting at least 3 of the 5 defects considered.

There are contradictory findings, in literature, regarding the possible cumulative effects of diverse oocyte morphological abnormalities on fertilization and embryo development.

An MII oocyte morphologic score was described by Rienzi et al. (2008) based on the impact that different oocyte morphological abnormalities have on fertilization rate and embryo morphology; a significant relationship was found between this score and clinical outcome; particularly they shown not only some intracytoplasmic abnormalities, but also particular extracytoplasmic abnormalities, are indicators of oocyte competence. On the other hand, Balaban et al. (1998) e Chamayou et al. (2006) reported no associations between oocytes morphology and fertilization rate, embryo quality and implantation rate.

The opposite findings for similar multiple morphologies suggest that distinct molecular biological mechanism having different effects on developmental potential of oocytes may exhibit a correlation with the same morphologies. Therefore, to reach a consensus on the multiple morphologies, more studies are required to find the signaling pathways showing direct or indirect interactions with these morphologies (Ozturk, 2020).

7.3 Relationship between oocyte morphology features and fertilization rate

We searched for a possible correlation between the single defect in oocyte morphology and the outcome of assisted fertilization. To do that, we considered only oocytes that had a single defect, the oocytes of score 4. The incidence of the various defects in this group of oocytes reflects the incidence of the entire population of oocytes examined in this study (Table 6): the defects more present are those of the cytoplasm and especially the presence of inclusions, then we find those of the vitelline space, especially the presence of inclusions in the space, and finally we find the defects of the polar globule that in most cases appears fragmented. Defects of shape and zona pellucida are rarer (Table 8).

FEATURES	
OOPLASM 159 OOCYTES (43,9%)	**GRANULAR** 42 OOCYTES (26,4%)
	CENTRALLY LOCATED GRANULAR AREA 15 OOCYTES (9,4%)
	INCLUSION 93 OOCYTES (58,4%)
	SER CLUSTERS 9 OOCYTES (5,6%)
SHAPE 9 OOCYTES (24%)	**IRREGULAR** 9 OOCYTES (100%)
PERIVITELLINE SPACE 94 OOCYTES (25,9%)	**LARGE** 12 OOCYTES (12,7%)
	INCLUSION 82 OOCYTES (87,2%)
I POLAR BODY 70 OOCYTES (19,3%)	**LARGE** 6 OOCYTES (8,5%)
	FRAGMENTED 64 OOCYTES (91,4%)
ZONA PELLUCIDA 34 OOCYTES (9,3%)	**THICK** 31 OOCYTES (91,1%)
	DARK 3 OOCYTES (8,8%)

Table 8. *Incidence of morphological defects in oocytes with score 4.*

Firstly, we analyzed the relationship between oocyte defects and fertilization rate (Table 9); the fertilization rate obtained for oocytes with no abnormalities was 71,9%, so the features that seem to influence fertilization the most are the presence of a centrally located granular area, the presence of granulations in the cytoplasm and a wide perivitelline space.

Then we compared the morphology of oocytes with the embryo development, in particular with cleavage rate and embryo quality rate, in particular with the cleavage rate and the embryo quality rate, and the characteristics that seem to influence these parameters are the same as those mentioned above (Table 10).

FEATURE		N° oocytes	2PN	%	*P value*
OOPLASM	GRANULAR	42	27	64,2%	NS
	CENTRALLY LOCATED GRANULAR AREA	15	8	53,3%	NS
	INCLUSION	93	73	78,4%	NS
	SER CLUSTERS	7	5	71,4%	NS
SHAPE	IRREGULAR	9	9	100%	.4
PERIVITELLINE SPACE	LARGE	12	7	58,3%	NS
	INCLUSION	82	62	75,6%	NS
I POLAR BODY	LARGE	6	4	66,6%	NS
	FRAGMENTED	64	49	76,5%	NS
ZONA PELLUCIDA	THICK	31	22	70,9%	NS
	DARK	3	2	66,6%	NS

Table 9. *Correlation between single oocyte feature and fertilization rate.*

OOCYTE SCORE	FEATURE		2PN	CLEAVAGE RATE	*P value*	GOOD QUALITY EMBRYO	*P value*
5			176	130 (73,8%)	NS	87 (49,9%)	NS
4	OOPLASM	GRANULAR	27	21 (77,7%)	NS	12 (44,4%)	NS
		CENTRALLY LOCATED GRANULAR AREA	8	4 (50%)	NS	1 (12,5%)	NS
		INCLUSION	73	61 (83,5%)	NS	48 (65,7%)	NS
		SER CLUSTERS	5	4 (80%)	NS	4 (80%)	NS
	SHAPE	IRREGULAR	9	8 (88,8%)	NS	6 (66,6%)	NS
	PERIVITELLINE SPACE	LARGE	7	5 (71,4%)	NS	3 (42,8%)	NS
		INCLUSION	62	54 (87%)	NS	47 (75,8%)	NS
	I POLAR BODY	LARGE	4	3 (75%)	NS	3 (75%)	NS
		FRAGMENTED	49	40 (81,6%)	NS	32 (65,3%)	NS
	ZONA PELLUCIDA	THICK	2	1 (50%)	NS	1 (50%)	NS
		DARK	22	19 (90,4%)	NS	17 (77,2%)	NS

Table 10. *Correlation between single oocyte feature, cleavage rate and embryo quality.*

Cytoplasmic texture appears to be a very important characteristics for oocyte evaluation, because this alterations may be a sign of oocyte immaturity (Kahraman et al., 2000).
Centrally located granular area is described as a more dense area than other regions in the center of the oocyte cytoplasm and considered to have an adverse effect on embryonic development (Kahraman et al., 2000). The correlation between this feature and developmental competence was discussed in literature, some reports a defective pronuclear morphology and reduced embryo quality (Ebner et al., 2008; Rienzi et al., 2008), in contrast ,in other study, oocytes with centrally located granulation had similar fertilizing and embryo development ability compared with a control group with normal morphology (Wilding et al., 2007).
Also Serhal et al. (1997) followed up the developmental outcome of oocytes with normal cytoplasm or presenting excessive granularity or cytoplasmic inclusions, while fertilization and grade I embryo rate were comparable between the three categories, pregnancy and implantation rates were found higher when the transferred embryos had developed from normal oocytes.
Although a recent study suggests that this type of granulation might be a normal manifestation of oocyte morphology. This hypothesis is based on the observation that oocytes with or without this defect had comparable developmental potential (Yi et al., 2019)

The other feature that seems to influence fertilization is the presence of a large PVS; the presence of this defect in the injected oocytes may be ascribed to an over maturity of oocytes (Mikkelsen & Lindenberg, 2001).
In literature large PVS is associated with a decreased fertilization rate (Rienzi et al., 2008); on the other hand it has been reported (Balaban et al., 1998; De Sutter et al., 1996) that there was no correlation observed between fertilization rate and large PVS. Furthermore, a study positively correlates enlarged space with embryo formation. (Hassan-Ali et al., 1998). Although there is no consensus on this parameter, it seems reasonable to prefer the oocytes having small PVS, but this does not mean that oocytes having large PVS cannot be inseminated.

7.4 Impact of semen quality

Although several studies have indicated a close relationship between oocyte and embryo qualities, the contribution of spermatozoa to early embryo development has been less clear. In order to evaluate the possible influence of poor seminal fluid quality on fertilization and embryo quality, we divided oocytes inseminated with seminal fluid with normal parameters from those inseminated with seminal fluid with altered parameters, as described in materials and methods. As can be seen in Table 11 and 12, oocytes inseminated with seminal fluid with altered

parameters had lower percentages of fertilization, cleavage rate and embryo quality rate than the other group.

It was shown a few years ago that fertilization rate, embryo quality, and pregnancy rates are inversely associated with a high number of immature spermatozoa and aneuploidy rates (Lazaros et al., 2011). Adverse paternal effect on oocyte development after insemination has been also described by Palermo et al. (1994) that suggested that this phenomenon may be mediated by the sperm centrosome dysfunction or deficiency of oocyte-activating factors.

Most studies to date have focused on the correlation between sperm DNA fragmentation, oxidative stress and IVF outcome; however, these tests are not performed routinely in fertility clinics therefore the value of the data may be limited in general practice. We performed a correlation analysis between the commonly analyzed semen parameters and fertilization and embryo quality rate, because we believe that this will provide valuable information to any IVF clinic for daily practice.

	NORMOZOOSPERMIA					**ALTERED SEMINAL PARAMETERS**				
SCORE	**N° OOCYTES**	**2PN**	**NO PN**	**% FERT**	***P value***	**N° OOCYTES**	**2PN**	**NO PN**	**% FERT**	***P value***
0	0	0	0	0	NS	1	0	1	0	NS
1	17	13	4	76,4	NS	18	12	6	66,6	NS
2	149	121	28	81,2	NS	77	61	16	79,2	NS
3	211	167	44	79,1	NS	173	124	49	71,6	NS
4	193	154	39	79,7	NS	169	114	55	67,4	NS
5	96	73	23	76	NS	150	104	46	69,3	NS
TOTAL	666	528	138	79,2	NS	588	415	173	70,5	NS

Table 11. *Correlation between oocyte score and fertilization rate in patient with normozoospermia and altered seminal parameters (at least one parameter lower than 5th percentile (World Health Organization, 2010)*

			NORMOZOOSPERMIA			ALTERED SEMINAL PARAMETERS		
OOCYTE SCORE	**FEATURE**		**2PN**	**EMBRYO**	**TOP QUALITY EMBRYO**	**2PN**	**EMBRYO**	**TOP QUALITY EMBRYO**
5			73	56 (76,7%)	33 (45,2%)	103	74 (71,8%)	61 (59,2%)
4	**OOPLASM**	**GRANULAR**	3	1	0	5	3 (60%)	3 (60%)
		CENTRALLY LOCATED GRANULAR AREA	13	10 (76,9%)	6 (46,1%)	14	11 (78,5%)	6 (42,8%)
		INCLUSION	46	39 (84,7%)	32 (69,5%)	27	22 (81,4%)	14 (51,8%)
		SER CLUSTERS	2	1	1	1	1	1
	SHAPE	**IRREGULAR**	4	3 (75%)	2 (50%)	3	3 (100%)	3 (100%)
	PERIVITELLINE SPACE	**LARGE**	4	4 (100%)	3 (75%)	3	2 (66,6%)	0 (0%)
		INCLUSION	31	26 (83,8%)	22 (70,9%)	31	28 (90,3%)	16 (51,6%)
	I POLAR BODY	**LARGE**	3	3 (100%)	3 (100%)	1	0	0
		FRAGMENTED	35	29 (82,8%)	22 (62,8%)	14	11 (78,5%)	10 (71,4%)
	ZONA PELLUCIDA	**THICK**	1	1	1	1	1	1
		DARK	10	9 (90%)	9 (90%)	12	8 (66,6%)	6 (50%)

Table 12. *Correlation between oocyte morphological feature and cleavage rate in patient with normozoospermia and altered seminal parameters (at least one parameter lower than 5th percentile (World Health Organization, 2010)*

7.5 Automatic analysis

7.5.1 Oocyte detection and identification of cytoplasmic abnormalities

At the end of the training, the network for the oocyte detection reached an accuracy value on the training of 0.94 and, in the test phase, was able to correctly identify eighteen oocytes out of twenty, with an accuracy of 90%.

A convolutional network was created to perform the classification of images containing cytoplasmic inclusions. After setting up the network, several datasets were identified and tested in order to assess whether the images previously generated with the help of the GAN architecture actually increased the performance of the classifier. In particular, four datasets were used:

- a dataset containing only the original augmented images, composed of 1,000 images of oocytes with inclusions and the same number of oocytes without inclusions. Of these 1600 (800 per class) were used for training and 400 (200 per class) for validation;

- a dataset containing only generated images, composed of one thousand synthetic images of oocytes with inclusions and as many without inclusions. Of these 1600 are used for training and 400 for validation;

- a dataset in which the training set consists only of synthetic images (800 with inclusions and 800 without inclusions), while the validation set consists of real images (400 images belonging equally to the two classes);

- a mixed training set composed of both synthetic images and real images (for each class 700 generated images and 300 real images) and a validation set containing only real images (200 images for each class).

The best result was obtained using the mixed dataset.

The tables show the accuracy values in the different experiment sets:

Batch\Learning rate	0.01	0.001	0.0001	0.00001
1	0.57	0.53	0.46	0.44
2	0.89	0.95	0.73	0.72
4	0.97	0.91	0.88	0.60
8	0.95	0.97	0.73	0.72

TABELLA 5: VALORI DI ACCURACY SUL TRAINING SET

Table 13. *Accuracy values on the training set.*

Batch\Learning rate	0.01	0.001	0.0001	0.00001
1	0.53	0.49	0.50	0.52
2	0.57	0.85	0.81	0.75
4	0.74	0.74	0.82	0.73
8	0.67	0.80	0.81	0.68

TABELLA 6: VALORI DI ACCURACY SUL VALIDATION SET

Table 14. *Accuracy values on the validation set.*

Batch\Learning rate	0.01	0.001	0.0001	0.00001
1	/	/	/	/
2	0.60	0.866	0.75	0.75
4	0.60	0.80	0.85	0.75
8	0.65	0.75	0.80	0.75

Table 15. *Accuracy values on the test set.*

It emerges, therefore, that despite the paucity of the original dataset, that required operations of creation of synthetic images and image augmentation, it was possible to train a deep network capable of classifying oocytes with cytoplasmic inclusions. Thanks to the use of synthetic images and image-augmentation techniques, the classifier achieved an accuracy of 86.66%. The tests carried out on the images generated by both the Pix2Pix and PGGAN architectures have shown that the networks were able to reproduce images of good quality, thus increasing the performance of the classifier. Similarly, the algorithm based on texture analysis, used for the identification of granular cytoplasmic granules, was able to correctly classify 91% of the oocytes and, in some cases, to correct our assessment based on the observation of microscopic features.

7.5.2 Prediction of fertilization outcome

The table shows the results obtained in both training and testing phases:

	Training 1	Training 2	Training 3	Training 4
Accuracy training	0.76	0.79	0.77	0.81
Accuracy test	0.69	0.71	0.66	0.70

Table 16. Results of anamnestic data classification (accuracy)

The classifier, after training, did not produce high performance. Since the performance of the MLP model used depend on the choice of descriptive features of the problem, the low performance obtained can be traced back to feature unbalance. In fact, our case study seems to be strongly unbalanced; for the IPB the normal category is the predominant one, while the increased size category (giant IPB) is present in just over ten oocytes. Therefore, in view of future applications of this classifier as a prediction tool, the population of oocytes should be enlarged so as to have a proper balance of all features. Furthermore, instead of using the categorization of seminal fluid, it may be useful to use the individual parameters of the seminal fluid analysis to obtain a more accurate prediction of the outcome of the fertilization process.

8 CONCLUSION

Assessment of oocyte quality is probably the most important and difficult task in IVF.
As an attempt to improve the results of ICSI cycles, it is important to identify and utilize non-invasive parameters able to predict oocyte quality. Considering the vital role played by the oocyte in the developmental process, selection criteria involving the stages preceding fertilization would be extremely useful in selecting embryos for transfer.
The aim of this study was to focusing on the assessment of oocyte morphology, with the aim of identifying features that could predict developmental competence Nonetheless, previous reports are conflicting regarding the effects of oocyte morphological abnormalities on fertilization rate and embryo quality rate. Our analysis demonstrates that the probability of an oocyte becoming fertilized is significantly reduced by the presence of a centrally located granular area, the presence of granulations in the cytoplasm and a wide perivitelline space. In accordance with Rienzi et al., (2008) we have shown that not only some cytoplasmic abnormalities, are indicators of oocyte competence. We believe that a more thorough assessment of oocyte morphological characteristics should be introduced into IVF laboratory practice.
This is why we have tried to develop an algorithm that can help embryologists to select the most competent oocytes for insemination in an automatic, objective and non-invasive way.
AI has long been utilized in other industries and has recently found a place in medical imaging; however, it is just beginning to have an impact on the clinical practice of reproductive medicine, a field familiar to rapid advancements and open to using new technologies to achieve the ultimate goal of a healthy baby.
Although Several studies have applied AI in ART, the limitations are often small datasets and the use of AI algorithms not specifically designed for the fertility clinic; large open datasets and methods specifically developed and tailored for use in context with ART could lead to better results and understanding. To significantly impact ART the AI-model must be developed in the context of the clinical practice but it is important to standardize the use of AI in ART to enable more transparent, comparable and reproducible results. To succeed with implementing AI as a valuable tool in the fertility clinic, a strong interdisciplinary collaboration is required between researchers in ART and AI as well as the clinical staff.

As a result of the experiments carried out, the approach used to create the application appears to be valid and the software developed has been judged useful by the experts of the clinic. Indeed, the application created will be subjected to further development. In the future, the use of a larger dataset could improve the performance of the networks and, in general, give rise to

new ideas for the implementation of other functionalities. The use of techniques for automatic detection of oocyte anomalies, as well as other tools for automating the processes used in clinics, would lead to a decrease in errors caused by human operators and, thus to a higher success rate of artificial insemination techniques.

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