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Non-invasive biomarkers of embryo quality: how they can contribute to the success of Assisted Reproduction Techniques

Settore Scientifico-Disciplinare: BIO/13, BIOLOGIA APPLICATA

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Introduction

1- Origins and evolution of Assisted Reproduction

Infertility is a global health issue affecting millions of people of reproductive age worldwide; it may occur due to male or female factors, a combination of both or may be unexplained.

Reproductive Medicine can be considered as a modern science; in fact, the first results date back to 1973 when Professors Carl Wood and John Leeton reported the first pregnancy in human In Vitro Fertilization (IVF) (De Kretzer D, 1973).

After treating a patient with clomiphene citrate for 5 days and inducing ovulation with human chorionic gonadotropin (HCG), the oocytes obtained by laparotomy were fertilized with the partner's sperm and about 74 hours after fertilization, an 8-cell embryo was transferred back to the patient's uterus. The pregnancy, unfortunately, resulted in an early miscarriage (De Kretzer D, 1973).

The turning point in the evolution of Assisted Reproduction occurs thanks to the studies of Robert Edwards and Patrick Steptoe. In 1976, they reported the second IVF pregnancy, which, however, resulted in an ectopic pregnancy (Steptoe PC, 1976). Edwards and Steptoe persisted and finally achieved their first live birth on with Louise Joy Brown, the first test tube baby born on July 25, 1978 (Steptoe PC, 1978). Sir Robert Geoffrey Edwards was awarded the Nobel Prize in Physiology or Medicine in 2010 for the development of In Vitro Fertilization. Today, there have been over 8 million births worldwide due to IVF, and this number is expected to grow.

1.1- Technological Advancements in IVF

The achievement of the first success of In Vitro Fertilization has opened up new perspectives in the field of Reproductive Medicine. Since infertility is a condition that afflicts a large number of people around the world, the possibility of finding a solution to this condition has aroused great interest in the development of new technologies; in fact, over the years, significant advances have been made around the world to improve the IVF process.

During the 1980s, there were increasing IVF cases of multiple gestations due to the high number of embryos being transferred. The idea of cryopreserving embryos would allow patients to save additional embryos for the future, thus decreasing the number of embryos transferred in the fresh cycle. In 1983, the Monash group reported the first cryopreservation of a human embryo using the slow freezing method (Trounson A, 1983). More specifically, Trounson and Mohr described the

techniques that would allow a four and eight cell embryo to survive the freeze, storage, and thaw process. However, the original slow rate freezing method was associated with low embryo post-thaw survival rate and, therefore, low pregnancy rates. This, with the high cost of cryopreservation, pushed others to search for better techniques.

In the following years, these problems were overcome with the introduction of the ultra-rapid freezing protocol (Trounson A, 1987). This technique, known as Vitrification, is based on the idea of applying a higher concentration of cryo-protectants and a rapid cooling speed to prevent the formation of intracellular ice crystals (Antinori M, 2010). Vitrification is faster to perform, offers better control of the process and, more importantly, offers a high post-thaw survival rate with improved pregnancy rates and live birth rates (Abdel Hafez FF, 2010).

Another important innovation in the field of IVF was the introduction of the Intracytoplasmic Sperm Injection (ICSI) by Gianpiero Palermo (Palermo et al. 1992), a new technique that has reported a significant breakthrough for male factor infertility. While attempting to perform sub-zonal injection of sperm for fertilization, Palermo noted that if the sperm was instead injected through the zona pellucida and into the ooplasm of the metaphase-II oocyte, fertilization would also occur. Thus, ICSI has become the standard of care for successfully treating severe male infertility cases.

In 1990, Cohen introduced a new concept to assist in implantation. At that time, the rate of embryonic implantation, or babies born per embryo transferred was <5%; Cohen's group hypothesized that a substantial number of healthy IVF embryos failed implantation due to inability to hatch from the zona pellucida (Cohen J 1990). Thus, they described piercing the zona pellucida with a microneedle until the needle tip was seen in the perivitelline space, a technique known as Assisted Hatching (Cohen J 1990). Since the technique was first described, there has been controversial debate of its contribution to increasing live birth rates. In 2012, the most recent Cochran Review showed an increased chance of achieving a clinical pregnancy with assisted hatching; however, there is still insufficient evidence on live birth rate (Carney SK, 2012). Thus, since Cohen's group first introduced this technique, fertility clinics today still continue to use this for specific patient populations.

As progress continued, the question emerged whether it was possible to screen embryos for inherited diseases. In 1990, Verlinsky and colleagues described the first polar body analysis of alpha-1-antitrypsin deficiency (Verlinsky Y, 1990). The authors removed, by micromanipulation, the first polar body from eight oocytes from a patient diagnosed with this disorder. Of these, six fertilized normally, showing removal of the first polar body did not affect subsequent fertilization or

the ability to grow to blastocyst stage. Successful PCR analysis was performed, and two embryos from the oocytes containing the unaffected gene were transferred. However, this attempt was not successful, since no pregnancy was obtained (Verlinsky Y, 1990). The main disadvantage of this technique is that it provides only genetic information about the maternal contribution with potential suboptimal diagnosis of aneuploidies. In 1999, the current standard for preimplantation genetic screening was described with trophectoderm biopsy (McArthur SJ 2005; De Boer K 2004; Xu K 1999). The first pregnancy following a blastocyst biopsy and Preimplantation Genetic Test (PGT) was in 2002 by De Boer's group (De Boer K, 2004). Since then, successful prevention of genetic diseases has been described.

The researchers continued to make dynamic advances in the journey that probably began centuries ago, and led to improvements in the modern medicine giving a ray of hope to the millions of infertile couples towards finding suitable treatment options for them. Thus, extensive refinement of techniques in the field of Assisted Reproductive Technology (ART) opens up opportunities in finding solutions to fertility problems for wider population, which previously were considered impossible to overcome.

2- From gametes to embryo: stages of human fertilization

Both male and female sex cells must pass through a long series of changes that convert them genetically and morphologically into mature gametes, which are capable of participating in the process of fertilization. Next, the gametes must be released from the gonads and make their journey to the upper part of the uterine tube, where fertilization normally takes place. Finally, the fertilized egg, now properly called an embryo, must enter the uterus, where it implants into the uterine lining.

2.1- Gametogenesis

Gametogenesis is typically divided into four phases: (1) the extraembryonic origin of the germ cells and their migration into the gonads, (2) an increase in the number of germ cells by mitosis, (3) a reduction in chromosomal number by meiosis, and (4) structural and functional maturation of the oocytes and spermatozoa. The first phase of gametogenesis is identical in males and females, whereas distinct differences exist between the male and female patterns in the last three phases (Figure 1).

- **Phase 1: origin and migration of germ cells**: primordial germ cells, the earliest recognizable precursors of gametes, arise outside the gonads and migrate into the gonads during early embryonic development. Germ cells exit from the yolk sac into the hindgut epithelium and then migrate through the dorsal mesentery until they reach the primordia of the gonads.
- **Phase 2: increase in the number of germ cells by mitosis:** after they arrive in the gonads, the primordial germ cells begin a phase of rapid mitotic proliferation. In a mitotic division, each germ cell produces two diploid progeny that are genetically equal. Through several series of mitotic divisions, the number of primordial germ cells increases exponentially from hundreds to millions. The pattern of mitotic proliferation differs markedly between male and female germ cells.

  Oogonia go through a period of intense mitotic activity in the embryonic ovary from the second through the fifth month of pregnancy in the human. During this period, the population of germ cells increases from only a few thousand to nearly 7 million. Soon after, numerous oogonia undergo a natural degeneration called “atresia”. Atresia of germ cells is a continuing feature of the histological landscape of the human ovary until menopause.

  Spermatogonia, which are the male counterparts of oogonia, follow a pattern of mitotic proliferation that differs greatly from that in the female. Mitosis also begins early in the embryonic testes, but in contrast to female germ cells, male germ cells maintain the ability to divide throughout postnatal life. The seminiferous tubules of the testes are lined with a germinative population of spermatogonia. Beginning at puberty, subpopulations of spermatogonia undergo periodic waves of mitosis. The progeny of these divisions enters meiosis as synchronous groups and this pattern of spermatogonial mitosis continues throughout life.
- **Phase 3: reduction in chromosomal number by meiosis**: the biological significance of meiosis is mainly due to the reduction of the number of chromosomes from the diploid (2n) to the haploid (1n) number, so that the species number of chromosomes can be maintained from generation to generation, and to the reassortment and redistribution of maternal and paternal genetic information through the process of crossing-over during the first meiotic division.

  Female meiosis ends with the formation of the secondary oocyte (arrested at metaphase), while male meiosis has mature sperm as its final product.

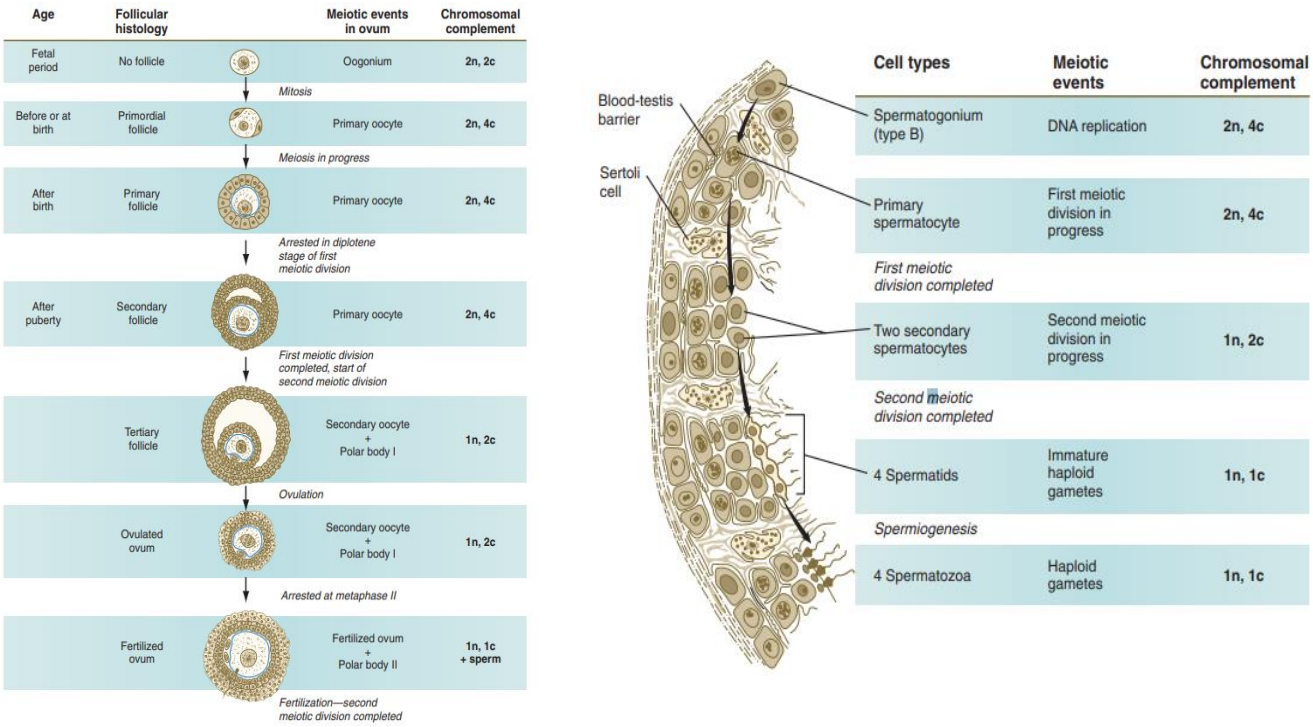

Figure 1: Summary of the major events in human oogenesis and spermatogenesis.

2.2- Fertilization and Preimplantation Development

Fertilization is a complex process that begin when spermatozoa start to penetrate the corona radiata that surrounds the egg and end with the union of the two gametes.

Once reached the oocyte, the sperm must pass through the zona pellucida for fertilization to occur and this is made possible by the acrosomal reaction; this process consists in the fusion of parts of the outer acrosomal membrane with the overlying plasma membrane, which results in the exocytosis of the lytic of enzymes that are stored in the acrosome.

After a brief transit period through the perivitelline space, the spermatozoon makes contact with the oocyte. Plasma membrane fusion of the two gametes leading to cell-cell continuity is the last step and leads to oocyte activation and zygote formation.

The zygote, which now has a diploid set of chromosomes, undergoes its first mitotic division and then continues to divide by mitosis into a number of smaller cells known as blastomeres.

The first event that determines the directed development of previously undifferentiated blastomeres is compaction, during which the individual outer blastomeres tightly adhere through gap and tight junctions and lose their individual identity when viewed from the surface. At about the third cleavage division there is a significant increase in RNA and protein synthesis, a marked change in the patterns of phospholipid synthesis, and the embryo undergoes compaction to form a morula.

Through the activity of a sodium/potassium ATPase-based sodium (Na+) transport system, Na+ and water (H2O) move across the epithelium-like outer blastomeres and accumulate in spaces among the inner blastomeres. This process, which occurs about 4 days after fertilization, is called cavitation, and the fluid-filled space is known as the blastocoele (blastocyst cavity). At this stage, the embryo is defined blastocyst.

At the blastocyst stage, the embryo, which is still surrounded by the zona pellucida, consists of two types of cells: an outer epithelial layer, the Trophoblast, that surrounds a small group of cells called the Inner Cell Mass (ICM). These two cell types represent the specialization of the blastomeres into two distinct cell lineages; indeed, cells of the inner cell mass give rise to the embryo and to several extraembryonic structures, whereas cells of the trophoblast form only extraembryonic structures, including the outer layers of the placenta.

To implant, a blastocyst must free itself from the protective zona pellucida, which becomes visibly thinner as the late blastocyst expands; hatching is completed within a few hours, and the freed blastocyst is separated from its empty zona (Figure 2).

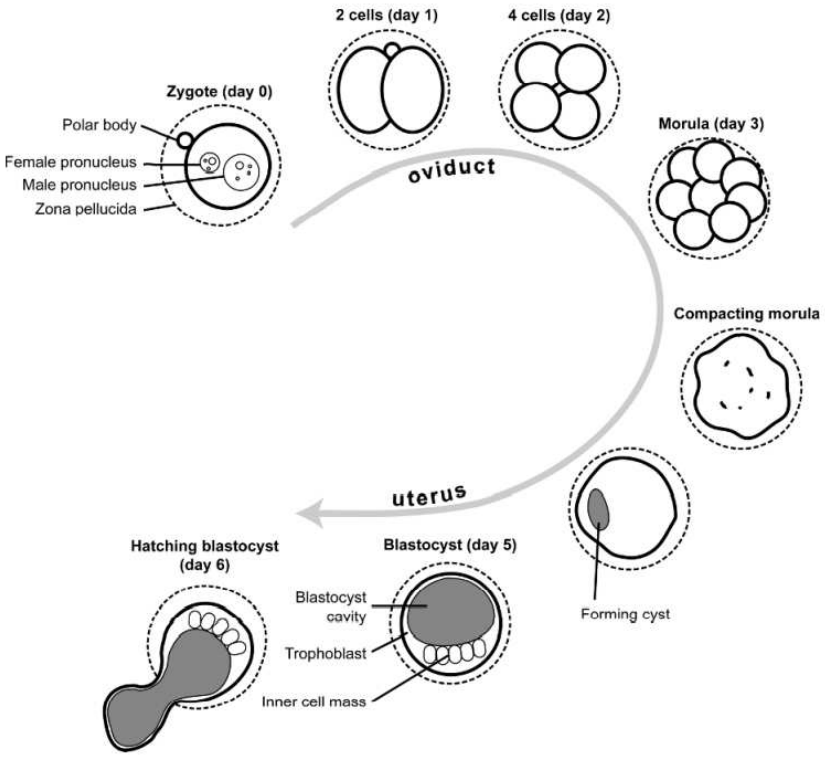

Figure 2: Preimplantation embryonic development. The zygote undergoes cleavage divisions as it travels down the oviduct towards the uterus.

3- Culture systems for the human embryo

Due to a considerable degree of plasticity, the human embryo may *in vitro* growth under a wide variety of culture conditions (Baltz JM, 2013); however, that ability to adapt to suboptimal culture conditions comes at the cost of impaired viability and potentially compromised pregnancy outcomes. The ability to develop even to the blastocyst stage, in fact, does not necessarily result in an healthy viable embryo, because it may appear morphologically normal, but altered at a cellular level (Ménézo et al, 1995).

At the early stages of development, prior to activation of the embryonic genome, the embryo possesses only limited capacity to maintain normal cellular functions such as regulation of intracellular pH (pHi), alleviation of oxidative stress and ionic homeostasis; so, a stress applied prior to compaction can affect embryo viability (Brinster RL, 1965; Quinn P, 1995). In contrast, the application of the same stress post-compaction and post-embryonic genome activation typically has limited negative impact on subsequent developmental competence (Lane et al, 2001; Lane et al, 2005; Gardner et al, 2015). For these reasons, maintenance of metabolic homeostasis at the early stages is essential for the maintenance of viability (Gardner et al, 2000).

In order to optimize embryo development *in vitro*, it is essential to consider and monitor all the parameters potentially affecting embryo culture, namely; culture media, supplements, culture dishes, incubation environment and gas phase, laboratory air quality, and even skills of the embryologists themselves.

The main stressors identified in the ART laboratory that can have a negative impact on gametes and embryos include transient temperature shifts, changes to the levels of CO_2 and in pH, physical stress, atmospheric oxygen, and the accumulation of ammonium from amino acids (Hardy et al, 1989; Dumollard et a, 2004; Gardner et al, 2005; Gardner et al, 1996).

Improvements in embryo culture media formulations have led directly to a significant increase in embryo implantation rates, thereby facilitating the routine introduction of single-embryo transfer (SET), which is considered the best practice for the majority of patients that seek IVF treatment. Today, culture media mainly consist of two types: sequential media, which provide the embryo with the nutrients it needs during the various stages of development, and Time-Lapse Media, based instead on the principle of letting the embryo choose what it needs during development (Gardner et al, 1998; Pool TB 2002).

This approach to single embryo transfer is also made possible through the introduction of Time Lapse Systems in the IVF laboratories; the benefits of time lapse culture, in fact, go beyond those of

embryonic selection alone, allowing the maintenance of stable and optimal culture conditions for the in vitro development of embryos.

4- Assessment of embryo morphology

Morphological evaluation of embryos has been the approach most commonly used by embryologists to assess embryo development and select the one/s to be transferred (Cummins et al, 1986). Since the beginning of human IVF, various grading systems based on numerous characteristics that can be observed in pre-implantation embryos have been evolved, with the aim of quantitating embryo development, viability and implantation potential. As embryo development is highly dynamic, the assessment at specific time points on each day has been adopted and key morphological characteristics of the embryo linked to developmental potential (Sakkas D, 2001).

The advantages of static morphological assessment are the simplicity and the limited expenses related to the method; there are, however, several limitations. The embryos are exposed to adverse effects of changes in temperature, humidity, and gas composition when removed from the incubator for inspection (Zhang et al. 2010). These effects becomes more prominent with increasing number of observations, making it necessary to limit the times in which embryos are removed from the incubator. Consequently, the evaluation becomes highly dependent upon the timing of the inspections (Montag et al. 2011, Scott et al. 2007) and the laboratory has to follow a fairly strict schedule for the assessment, which in turn results in a less flexible workflow. Finally, aneuploidy, which is the most significant reason for implantation failure, is very poorly reflected in the human embryo's morphology (Alfarawati et al. 2011, Ziebe et al. 2003). Cell-cycle checkpoints that minimize the risk of chromosomal errors do not function as well in the gametes and early pre-implantation embryos compared with somatic cells; this offers an explanation to why chromosomally abnormal embryos may continue a development similarly to the viable embryos but fail to establish a pregnancy. The lack of a clear association between the embryos chromosomal constitution and morphological appearance may pose the most critical hindrance for achieving higher implantation rates based on morphological embryo selection.

While molecular and biochemical methods to assess human gamete reproductive potential and embryo quality have been described and are increasingly utilized, these methods are still relatively complex, time-consuming, and expensive for most ART laboratories. Hence, the assessment of morphology has been, and still remains, the most widely employed method for embryo selection as

it is quick, non-invasive, easy to carry out in routine practice, inexpensive and, although not without faults, has consistently been shown to have predictive value.

Each stage of the complex developmental process is dependent upon the successful completion of the previous one. Consequently, the optimal evaluation of an embryo's potential would require multiple assessments at each developmental checkpoint as it makes its complex transition from a single-celled zygote to a blastocyst (Figure 3).

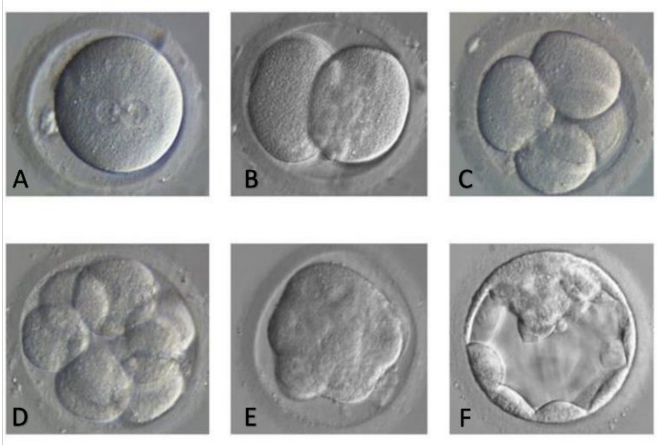

Figure 3: Different stages of the embryo development. A: zygote; B: two blastomeres; C: four blastomeres; D: eight blastomeres; E: morula; F: blastocyst.

A comprehensive morphological evaluation should assess the end result at each checkpoint but also the path taken to reach that point.

The first step of embryo morphology assessment is the fertilization check, occurring 16–18 hours after conventional insemination or intra-cytoplasmic sperm injection (ICSI); normal fertilization is confirmed by the presence of two pronuclei.

Over the years, many scoring systems have been developed to classify the zygotes according to some main characteristics of the two pronuclei (symmetry, position, location, or number and distribution of the nucleolar precursor bodies), but evidence-based data show that changes in pronuclear stage are highly time-dependent. Despite initial promise for the predictive value of pronuclei assessment, subsequent studies have been inconsistent and multiple groups failed to demonstrate correlation between pronuclear assessments and pregnancy rates (Payne D et al, 1997; Salumets et al, 2001; Jaroudi et al, 2004).

Researchers have been able to develop multiple scoring systems to assess cleavage embryos on days 2 and 3, most focus on the presence/absence of multinucleation, fragmentation, and blastomere

number, size and morphology as these parameters have been found to be most predictive of subsequent development and favourable transfer outcomes (De Neubourg et al, 2004; Van Royen et al, 2003).

Timing of the first cell division of the embryo has been investigated as a predictor of developmental competence, with the suggestion that "early cleavage" is associated with higher pregnancy rates. However, there is only a certain window where true early cleavage can be seen, and this extra assessment may be difficult to fit into the normal routine of an IVF laboratory.

According to the Istanbul Consensus on embryo assessment (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011), on average, embryos that have cleaved more slowly than the expected rate are likely to be abnormal and have a reduced implantation potential; in fact, some studies have shown that when transferring single embryos, a significantly higher clinical pregnancy rate was observed after transfer of early-cleaving (50%) rather than non-early cleaving (26.4%) embryos.

Multinucleation, defined as more than a single interphase nucleus, is a key morphological feature of the cleavage stages, and its presence is associated with reduced viability and implantation potential; furthermore, multinucleated embryos are associated with an increased level of chromosome abnormality and, as a consequence, increased risk of spontaneous abortion. The grading scheme for multinucleation should be binary, noting its presence or absence (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Embryo fragmentation is a result of the expulsion of anucleate, membrane-bound cytoplasm that occurs during embryo cleavage and is associated with aberrant cell division (Abeyta et al, 2014; Puissant et al, 1987; Alikani et al, 1999; Alikani et al, 2005). These fragments have been argued to be both by-products and catalysts of apoptosis (Jurisicova et al, 2003; Perez et al, 1999) or an indicator of the irregular loss of synchrony between nuclear and cytoplasmic cell division. While it is difficult to demonstrate whether this is a consequence or precursor of fragmentation, surviving blastomeres may be depleted of critical organelles, substrates, or signal ligands (Browne et al, 2009; Lin et al, 2004) as a result of excessive fragmentation affecting future development and reproductive potential.

Extensive fragmentation is known to be associated with implantation failure and poorer blastocyst formation rates, but the relationship between the degree of fragmentation and the developmental potential of the embryo is far from clear.

Assessment of fragmentation is typically quantified as the percentage of the embryo the fragments occupy (Antczak M, 1999). The relative degrees of fragmentation were defined as: mild (<10%);

moderate (10–25%) and severe (>25%). Recently, with the advent of time-lapse imaging, fragment reabsorption and the presence of chromosomes within fragments have been demonstrated (Abeyta et al, 2014; Chavez et al, 2012) indicating that the interpretation of fragmentation may be more complex than simply its presence or absence.

Mitosis results in an even number of cells with the cytoplasm equally split between daughter cells, resulting in even numbers of similar-sized cells, which decrease in size with each round of cell division. This ideal is reflected in many cleavage-stage embryo grading systems which extrapolate that uneven distribution of organelles, proteins, and RNA parallel asymmetric division and may be detrimental to embryo development (Steer et al, 1992; Desai et al, 2000; Hnida et al, 2004). Despite this concept, slight asymmetry at a random static observation is normal in human development that results in transient 3-, 5-, and 7-cell embryos to allow for cell spacing as the embryo divides (Roux et al, 1995).

An ideal scoring system for cleavage-stage embryos, therefore, should consider cell number and size, grade of fragmentation and presence of multinucleation; the consensus scoring system is presented in Table 1.

GRADE	RATING	DESCRIPTION
1	*Good*	< 10% fragmentation Stage-specific cell size No multinucleation
2	*Fair*	10-25% fragmentation Stage-specific cell size for majority of cells No evidence of multinucleation
3	*Poor*	Severe fragmentation (> 25%) Cell size not stage-specific Evidence of multinucleation

Table 1: Consensus scoring system for cleavage-stage embryos (in addition to cell number).

Compaction of an embryo is one of the key developmental events during the preimplantation period and has a profound effect on how the embryo develops in culture (Tao et al, 2002; Skiadas et al, 2006; Nikas et al, 1996).

During compaction, the blastomeres establish tight junctions to facilitate the formation of the first transporting epithelium of the conceptus and cytoplasmic polarization begins to appear as a result of embryo transcription (Feil et al, 2008).

The blastocyst represents the ultimate stage of clinical embryo culture before transfer back into the uterus, and therefore it is the final stage at which morphological assessment is possible. As with the scoring of embryos during the cleavage stages, time and morphology are key in selecting the best blastocyst.

A blastocyst is defined by the onset of cavitation and the formation of the blastocoel which coincides with cell differentiation between the Inner Cell Mass (ICM) and Trophectoderm (TE) (Hardarson et al, 2012). These defining aspects of the blastocyst have become the focal points of morphological assessment systems which typically focus on the degree and timing of expansion, the quality of the inner cell mass, and the trophectoderm.

The scoring assessment for blastocysts devised by Gardner and Schoolcraft (Gardner et al, 1999) is one of the most widely adopted; it is a comprehensive alphanumeric system, designed to incorporate the degree of expansion, as well as the quality and cell number of the two cell lineages.

Blastocysts are graded on the basis of volume/expansion as (Figure 4):

- Grade 1- Early blastocyst: the blastocoele occupies less than half the volume of the embryo.
- Grade 2- Blastocyst: the blastocoele occupies half the volume of the embryo or more.
- Grade 3- Full blastocyst: the blastocoele completely fills the embryo, but the zona has not thinned.
- Grade 4- Expanded blastocyst: the volume of the blastocoele is larger than that of the ICM, and the zona is thinning.
- Grade 5- Hatching blastocyst: the trophectoderm has started to herniate through the zona.
- Grade 6- Hatched blastocyst: the blastocyst has completely escaped from the zona.

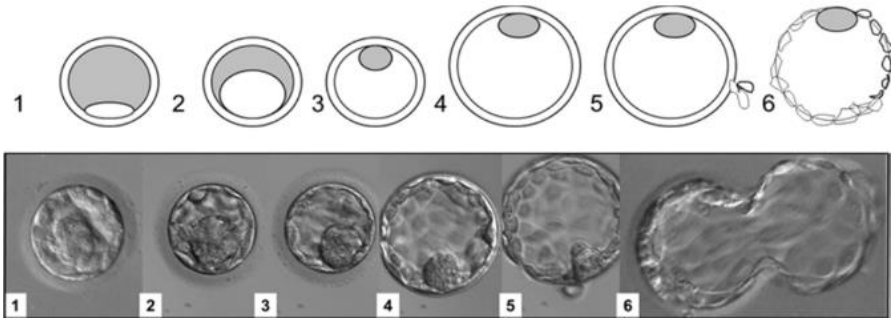

Figure 4: Different grades of blastocyst expansion. 1: early blastocyst; 2: Blastocyst; 3: Full blastocyst; 4: Expanded blastocyst; 5: Hatching blastocyst; 6: Hatched blastocyst.

The inner cell mass becomes clearer as the blastocoel expands. These cells, which will become the fetus and extraembryonic structures, can have morphology ranging from very large tightly packed cells to loosely aggregated smaller cells (Hardarson et al, 2012; Embryology ASiRMaESIGo, 2011; Schoolcraft et al, 1999).

ICM can be scored as (Figure 5):

- Grade A: tightly packed, many cells.
- Grade B: loosely grouped, several cells.
- Grade C: very few cells.

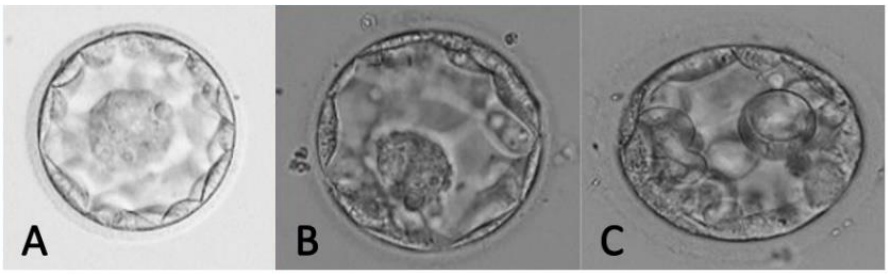

Figure 5: Inner Cell Mass scoring. A: tightly packed, many cells; B: loosely grouped, several cells; C: very few cells.

The trophectoderm represents the cells in direct contact with the endometrium responsible for apposition, adhesion, and invasion of the endometrium and eventually becomes the extra-amniotic membranes and placenta (Gardner et al, 1999). A larger number of TE cells will be able to ensure greater signalling and interaction with the endometrium.

Similar to the ICM, the TE can be evaluated by the number of cells and relative cohesiveness with each other as follows (Figure 6):

- Grade A: many cells forming a cohesive epithelium.
- Grade B: few cells forming a loose epithelium.
- Grade C: very few large cells.

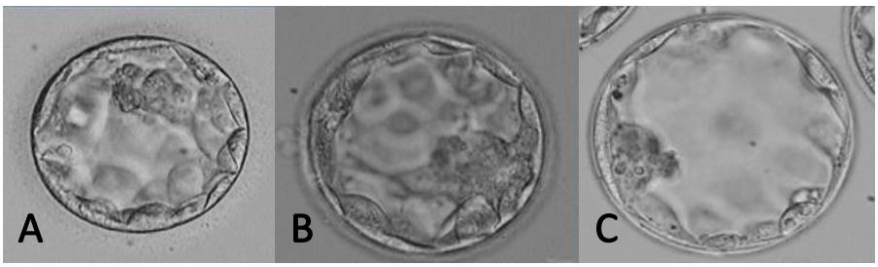

Figure 6: Trophectoderm. A: many cells forming a cohesive epithelium; B: few cells forming a loose epithelium; C: very few large cells.

The significance of examining the embryo post compaction is the ability to examine it after activation of the embryonic genome. Furthermore, the obvious benefit of looking at the blastocyst is the ability to examine both ICM and TE; the extent to which the TE develops will reflect the embryo's ability to attach and implant in the endometrium, while development of the ICM is obviously crucial for the development of the fetus itself (Kovacic et al., 2004).
The potential advantages of blastocyst culture and transfer, therefore, include:

1. Synchronizing embryonic stage with the female tract. This is important as the levels of nutrients within the fallopian tube and uterus do differ, and therefore the premature transfer of the cleavage-stage embryo to the uterus could result in metabolic stress (Gardner et al, 1998). Furthermore, the uterine environment during a stimulated cycle cannot be considered normal.
2. When embryos are selected for transfer at the two- to eight-cell stage, the embryonic genome has only just begun to be transcribed (Braude et al, 1988; Taylor et al, 1997), and therefore it is not possible to identify from within a given cohort those embryos with the highest developmental potential. Only by culturing embryos past the maternal/embryonic genome transition and up to the blastocyst does it become realistic to identify those embryos with limited or no developmental potential.

3. Many abnormal embryos arrest during development in vitro, so by culturing embryos to the blastocyst stage, one has already selected against those embryos with little if any developmental potential.
4. Cryopreservation of embryos at the blastocyst stage appears more successful than at earlier stages (185).
5. Trophectoderm biopsy and analysis enable the removal of more cells compared to cleavage-stage embryos, which facilitates the use of newer technologies (Fragouli et al, 2008; Schoolcraft et al, 2010).

In summary, careful evaluation of embryo morphology will detect dysmorphic and arrested embryos, at least 50% of which are chromosomally abnormal, which should not be transferred if morphologically better embryos are available. However, this evaluation does not allow selection against aneuploidy, the incidence of which in normal embryos increases from 30% in women 35-39 years of age to over 60% in women older than 40 (Munne´ et al., 2007). Culture to blastocyst stage eliminates more post-meiotic abnormalities, but not aneuploidy. The remainder of chromosomal abnormalities can only be identified through preimplantation genetic diagnosis.

5- Time Lapse Technology

The introduction of Time Lapse Microscopy (TLM) as a clinical tool in IVF has resulted in several reports of improved embryo selection based on the analysis of sequential time lapse images of embryo development. Time lapse devices allow the capture of images of the embryos developing in vitro at regular intervals throughout the culture period; this provides continuous and uninterrupted monitoring that furnishes embryologists with a more sophisticated and promising tool for the study and selection of the human preimplantation embryo beyond conventional daily microscopy (Figure 7).

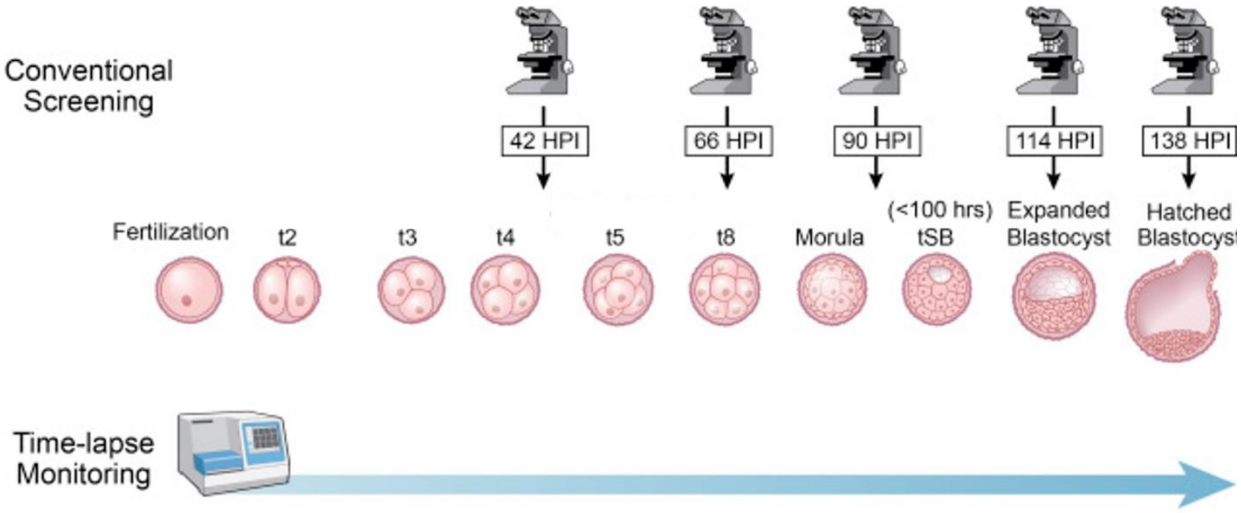

Figure 7: Embryo development over time. The top of the figure shows the conventional daily observations (HPI: hours post insemination) while Time-lapse monitoring occurred continuously.

A Time Lapse Technology (TLT) system consists of an incubator with integrated microscope and cameras connected to an external computer. Embryo images are captured at defined time intervals and on different focal planes for the entire duration of the culture. The acquired images are collected into a video which allows a detailed morphological and morphokinetic evaluation of all the validated parameters without interrupting embryo culture and eliminates the restriction of rigid time-schedules for observation (Figure 8).

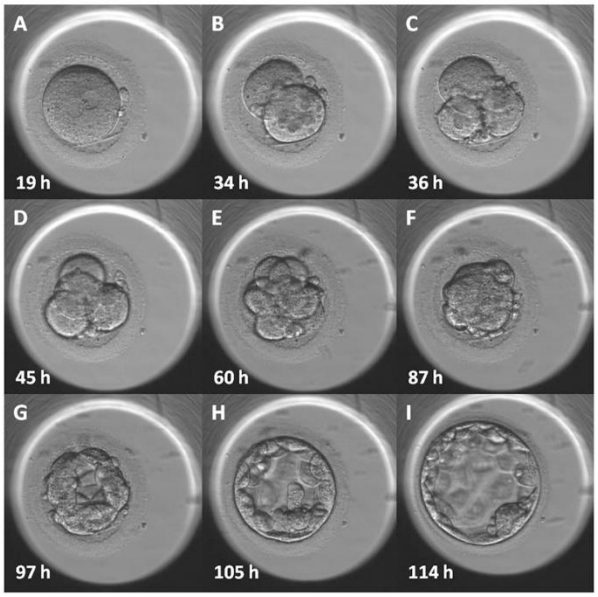

Figure 8: Stages of early human embryo development taken from a time-lapse image series showing the embryo at nine representative time-points. A: oocyte at pronuclear stage at 19 hours; B: 2-cell stage at 34 hours; C: 3-cell stage at 36 hours; D: 4-cell stage at 45 hours; E: 8-cell stage at 60 hours; F: morula stage at 87 hours; G: early blastocyst stage at 97 hours; H: blastocyst stage at 105 hours; I: expanding blastocyst at 114 hours.

A key element of all of the systems is the manual or semi-automated interpretation of events and recording (or annotating) of the specific points in preimplantation development. These annotations and calculations can then be used prospectively as additional information in the choice of embryo for transfer or freezing.

The use of TLM systems in the IVF laboratories brings the embryologists an advantage to visualize the whole biological development events and thereby to better determine the most viable embryo for implantation (Chen et al, 2013; Ciray et al, 2014); however, there are other advantages that do not only concern the possibility of carrying out the morphokinetic assessment of the embryo development:

- Stable culture system: undisturbed culture environment and minimized the undesirable risks for developing embryos.
- Embryo de-selection: possibility to exclude embryos that display aberrant cleavage patterns impossible to see using a static evaluation.
- Improved workflow in the IVF laboratory: no need to assess embryos at fixed time points.

- Improvement in quality control and quality management: high degree of intra- and inter-observer agreement.
- Possible use of algorithms for embryo selection: search for morphokinetic biomarkers that allows to develop special algorithms and formulas for cumulative embryo evaluation.

5.1- Morphokinetics

Failure to select a viable embryo from a cohort will inevitably limit the chance of a positive outcome. The unwitting selection of a nonviable embryo, and the cryopreservation of viable alternatives within a cohort, will also delay the desired outcome.

Morphokinetic, which is defined as the combination of the embryo appearance (morphology) and the timing in which cellular events occur (kinetics), enables to assign a precise time (numeric parameter) to every aspect of embryo development, starting as early as extrusion of the second polar body and continuing up to the time of hatching at the blastocyst stage (Montag et al, 2016). Of note, time lapse imaging was able to detect some events, which are clearly linked to embryo viability, but were previously unknown due to the limitations imposed by standard embryo assessment at fixed time points.

When using a time lapse system, the most widely recorded morphokinetic parameters are the pronuclear (PN) appearance (tPNa) and fading (tPNf) and cellular division from 2- to 9-cell or more (t2, t3, t4, t5, t6, t7, t8, t9+). The rounds of cleavage are also considered: times between 2- and 3-cell stages (cc2, second round calculated as t3-t2), between 3- and 5-cell stages (cc3, third round calculated as t5-t3) and between 5- and 9-cell stages (cc4, fourth round calculated as t9-t5). In addition, TLT allows to assess the synchrony of cellular divisions defined as s2 (calculated as t4-t3) and as s3 (calculated as t8-t5). Finally, the timing of starting compaction (tSC), morulae formation (tM), starting blastulation (tSB), full blastocyst stage (tB or tFB), blastocyst expansion (tEB), and hatching (tHB) are annotated. The time of intracytoplasmic sperm injection (ICSI) is usually considered as the starting time point (t0), and values are expressed as hours post insemination (hpi).

5.2- Future perspectives of TLT

Currently, research projects concerning the development of new models are ongoing. By analyzing videos from thousands of embryos, time lapse microscopy should allow to create an ideal prediction model without pre-existing assumptions.

One perspective in the use of time lapse technology is the introduction of automation for embryo assessment. This will improve not only quality control and traceability but also general efficiency and productivity of different procedures. Applied to clinical embryology, automated image analysis is particularly challenging due to several unique features of the developing human embryo.

The search for objective robust biomarkers of embryo viability continues, although to date, and despite significant research effort, no single reliable biomarker with a sufficiently high predictability of live birth has yet been identified: this may reflect the complexity of preimplantation development. Consequently, the search for biomarkers must no longer occur in isolation; the combination of TLT with other markers of embryo physiology is a natural evolution of both fields.

6- The non-invasive quantification of embryo physiology

In recent years, new methods have been developed involving the analysis of embryo physiology to determine its viability; the assessment of physiological traits, such as nutrient utilization and metabolic activity, or the production of embryo-specific factors, may in fact be able to identify the healthiest embryos within a transfer cohort.

A number of quantitative techniques have been trialled that attempt to monitor the uptake of specific nutrients by the embryo from the surrounding medium, and to detect the secretion of specific metabolites and factors into the medium.

These methods aim to measure changes in culture media and to fulfill three key criteria so that they can be applicable in IVF clinics:

1. they must have the ability to measure the change without damaging the embryo.
2. they must have the ability to measure the change quickly.
3. they must have the ability to measure the change consistently and accurately.

Several studies using a variety of methods have correlated the turnover of specific individual metabolites such as glucose, pyruvate, and amino acids to developmental potential and viability

(Brison et al, 2004; Conaghan et al, 1993; Gardner et al, 2001, 2011; Hardy et al, 1989; Houghton et al, 2002). Such methods are powerful because they yield quantitative data on consumption or production of substrates under investigation. These approaches are, however, indirect, meaning that while we can measure depletion or appearance of compounds, we cannot easily track the fate of each molecule through the various pathways available to it. An added complication to performing non-invasive assays is that many commercially available media are not suitable as substrate concentrations are too high to detect changes. However, this feature is a challenge for the translation of physiology-based assays into the clinical situation.

As already assumed several years ago, it is desirable that this problem can be overcome through the development of microfluidic devices, capable of manipulating and analysing sub-microlitre samples of human embryo culture medium (Gardner et al, 1994).

Finally, the use of metabolomic and proteomic platforms has yet to be proven and employed clinically, analysis of embryo function can greatly enhance our understanding of development, and hence such approaches could ultimately assist in defining parameters that can be used in embryo selection. To this end, an analysis of the relationship between the morphokinetic development of embryos and their metabolic activity has been undertaken.

6.1- Extracellular Vesicles as possible biomarker of embryo quality

In recent years, several fields of biomedical research have been interested in the study of extracellular vesicles (EV) as biomarkers for diagnosis and monitoring of various, not necessarily pathological conditions (Vlassov et al, 2012) (Figure 8).

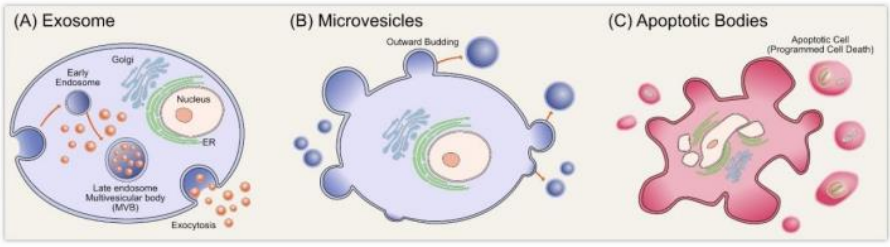

Figure 8: Representation of different types of extracellular vesicles.

Among the different types of EVs, exosomes are commonly defined by their nano-size (30-100 nm) and biogenesis pathway having origin in the cell's endosomal compartment; they originate from the endosomal compartment of the cell and are shown to contain material characteristic of the cell of origin, that could be proteins, lipids or nucleic acid.

Exosomes have become a hot topic of clinical research because of their extensiveness, simplicity, and non-invasiveness, as they can be also extracted from readily accessible body fluids paving the way to future noninvasive diagnostic models (Hou et al, 2019).

Recently, extracellular vesicles and exosomes have attracted significant attention in the reproduction field due to their various roles in gametogenesis, oocyte selection, semen quality diagnostics, embryo selection, detection of endometrial receptivity including embryo-endometrial dynamic cross-talk. Them comprise subpopulations differing in size, morphology, composition and mechanisms of biogenesis (Simon et al, 2018).

EVs can vary widely in terms of composition and may carry specific sets of species derived from the type of cell of origin and determining their fate and functions. Extracellular vesicles can be nowadays studied as biomarkers for several reproductive conditions and, consequently, some of the accumulated knowledge may be translated to clinical practice to inform assisted reproduction diagnostics (Giacomini et al, 2020).

Several data demonstrated that EVs are one of the most critical components that are involved in cell-to-cell communication and different studies demonstrated the production and secretion of EVs by mouse and porcine embryos. (Burns et al, 2014; Ruiz-Gonz´alez et al, 2015; Desrochers et al, 2016).

During the in vitro fertilization cycles, human embryos are routinely cultured resulting as opportunity to investigate their secretory products, especially since these types of analysis on media culture does not offer any risk to the embryo or compromise the patient's treatment success.

Research study purpose

Since the beginning of human IVF, various grading systems based on numerous characteristics that can be observed in pre-implantation embryos have been evolved, with the aim of quantitating embryo development, viability and implantation potential. The advantages of static morphological assessment are the simplicity and the limited expenses related to the method; there are, however, several limitations as it doesn't allow to evaluate events that occur during *in vitro* development which are fundamental for determining embryo quality.

The introduction of Time Lapse Microscopy in the field of Assisted Reproduction allowed the identification of parameters that may non-invasively predict the developmental potential of a cleavage-stage embryo through continuous monitoring; the capture of images of the embryos developing *in vitro* at regular intervals throughout the culture period, in fact, provides embryologists with a more sophisticated and promising tool for the study and selection of the human preimplantation embryo. However, to date, and despite significant research effort, no single reliable biomarker with a sufficiently high predictability of live birth has yet been identified. Consequently, the search for biomarkers must no longer occur in isolation and the combination of TLT with other markers of embryo physiology is a natural evolution of both fields.

In recent years, new methods have been developed involving the analysis of embryo physiology to determine viability. The use of metabolomic and proteomic platforms has yet to be proven and employed clinically, and hence such approaches could ultimately assist in defining parameters that can be used in embryo selection. An example of a non-invasive assessment of embryo quality can be represented by the analysis of EVs in the spent culture media; extracellular vesicles, in fact, can be nowadays studied as biomarkers for several reproductive conditions.

The proposal of this thesis was to evaluate how the morphokinetic assessment of embryo development can contribute to the success of Assisted Reproduction treatments. Data obtained through the record of cellular divisions parameters of embryos incubated in a time lapse system, were retrospectively analyzed according to different outcomes of IVF treatments in order to identify markers that can provide more information on embryonic quality and, consequently, can guide the embryologist in the selection of embryos with the greatest development and implantation potential in a non-invasive way.

At the same time, a preliminary analysis was started on the spent culture media of the embryos cultured *in vitro* with the aim of evaluating the presence and quantity of extracellular vesicles (EVs) that can be considered non-invasive biomarkers able to provide additional information on embryo quality.

Materials and Methods

1- Study sample

In the present study were included embryos from 97 patients who underwent assisted reproduction treatments between September 2018 and December 2021 at the IVF center A.G.I. Medica, Siena. All women have signed a written informed consent. Exclusion criteria considered in this study were: low response (less than five metaphase II oocytes), polycystic ovary syndrome (PCOS), hydrosalpynx, BMI> 30 kg/m^2 and BMI< 19 kg/m^2 , uterine pathology (myomas, adenomyiosis, trombophylia, chronic pathologies, acquired or congenital uterine abnormalities), maternal age over 45 years old (aging uterus), or severe male factor (less than 5 million motile sperm cells in total in the ejaculate).

The retrospective morphokinetic analysis was carried out on a total of 369 embryos that were incubated in the Miri TL® (Esco Medical, Egaa, Denmark) time lapse system; all embryos were obtained after fertilization by ICSI and were investigated by detailed time lapse analysis measuring the exact timing of the developmental events in hours post insemination. Embryos were selected for the analysis based on the achievement of the blastocyst stage (n= 255), the outcome of the treatment (positive n= 26; negative n= 71) and the state of euploidy (n= 37) or aneuploidy (n=40) ascertained by the Preimplantation Genetic Test (PGT).

2- Oocyte pick-up and ICSI

The controlled ovarian stimulation consisted of short GnRH-agonist protocol followed by stimulation with gonadotrophins. The daily dose of FSH was adjusted according to the patient's ovarian response based on serum estradiol levels and the number and size of ovarian follicles as considered by transvaginal ultrasonography; a single injection of hCG was administered to induce final follicular maturation. Oocyte retrieval was performed by transvaginal ultrasound-guided follicle aspiration 36 hours after hCG injection.

After sperm preparation, the oocyte-cumulus complexes were denuded using hyaluronidase and ICSI was performed on mature oocytes following the procedure described by Palermo (Palermo et al, 1992).

3- Embryo culture

After micro-injection, inseminated oocytes were immediately transferred into pre-equilibrated CultureCoin® (Esco Medical, Egaa, Denmark) with 30 µl of culture medium (Continuous Single Culture® Complete. FUJIFILM Irvine Scientific, Santa Ana, California) under oil (Oil for Embryo Culture. FUJIFILM Irvine Scientific, Santa Ana, California). The slides were prepared and left in an incubator to pre-equilibrate at 37°C and 6% CO_2 atmosphere. After pre-equilibration, all air bubbles are meticulously removed before the inseminated oocytes are placed individually in CultureCoin® microwells and incubated in the time lapse monitoring system at 37°C in 6% CO_2 concentration and 5% O_2 concentration until embryo transfer.

The time lapse instrument Miri TL® (Esco Medical, Egaa, Denmark) is a tri-gas incubator with a built-in microscope to automatically acquire images of up to 84 individual embryos during culture: embryo development was recorded every 15 minutes in five different focal planes and the images and related data were stored in the MIRI® TL Viewer software (Esco Medical, Egaa, Denmark) and subsequently analysed. Embryos were selected for transfer according to the guidelines proposed by the Istanbul Consensus on embryo assessment (2011).

For embryos that underwent PGT, trophectoderm biopsy was performed on the fifth or sixth day of *in vitro* development; results for all samples were reported as ‘‘euploid” when no chromosome abnormality was detected, or ‘‘aneuploid’’ when the chromosomal status was altered.

The β-hCG value was determined 13 days after embryo transfer and the clinical pregnancy was confirmed when a gestational sac with fetal heartbeat was visible by ultrasound examination.

4- Time-lapse evaluation of morphokinetic parameters

Retrospective analysis of the acquired images of each embryo was made with an external computer, MIRI® TL Viewer software (Esco Medical, Egaa, Denmark), using an image analysis software in which all the considered embryo developmental events were annotated together with the corresponding timing of the events.

The time of ICSI is usually considered as the starting time point (t0) of TL monitoring and the values of the events observed during embryo development are expressed as hours post insemination (hpi).

Successful fertilization was assessed at 16–18 hours post-ICSI and confirmed by the presence of two pronuclei and two polar bodies; pronuclear appearance was reported as “tPNa”, referring to the first frame where both the male and female pronucleus are visible and juxtaposed. Pronuclear

disappearance was instead reported as "tPNf" (time of pronuclear fading) and annotated at the first frame in which both pronuclei were no longer visible.

The timings of cleavage from 2 to 9 and more cells (t2, t3, t4, t5, t6, t7, t8, and t9) were annotated at the first frame in which the blastomeres were seen as separated by individual membranes; time of compaction (tComp) was instead defined as the stage at which it was no longer possible to visualize the individual blastomere membranes, until the formation of the morula (tM).

Time to early blastocyst (tEaB) was annotated when the blastocoele cavity filled less than half the volume of the blastocyst, while time of blastocyst (tB) consisted of the time when the cavity filled the embryo and the inner cell mass and trophectoderm tissues were distinguishable from each other.

The annotation of tEB (time of expanded blastocyst) was defined by the time frame after the zona pellucida starts to thin and, finally, timing of hatching of the blastocyst (tHB) was reported when the trophectoderm starts herniating through the zona pellucida.

5- Nanoparticle Tracking Analysis (NTA)

NTA was performed on embryo spent media using a NanoSight LM10-HS microscope (NanoSight, Ltd., Amesbury, UK) equipped with a 405 nm laser. Samples were diluted with PBS to reach a working concentration of 20–120 nanoparticles per frame and/or 107–109 nanoparticles/mL. The threshold was set at 5, and the camera level 16 was used, in line with the preferential detection of 50-200 nm particles. For each sample, three 30-sec videos with a frame rate of 30 frames per second were recorded. Temperature was monitored and recorded throughout the measurements. Captured videos were analyzed by NTA software (version 3.2) (Malvern Panalytical, Herrenberg, Germany) to determine nanoparticle concentration and size with relative standard error. For analysis, automatic settings were used for blur, minimum track length, and minimum expected particle size. Prior to analysis, calibration of the NanoSight system was performed using polystyrene latex microbeads with a size of 100, 200 nm, and 400 nm (NanoSight, Ltd.).

6- Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). The time variables were tested for normality using Shapiro-Wilks test for normality. Since most variables were found not to be normally distributed, non-parametric tests were used to determine whether differences were significant. The Mann-Whitney U test was used to test differences in morphokinetic variables. Differences were considered as statistically significant with p-value ≤ 0.05.

To describe the distribution of the probabilities of implantation, timings were converted from continuous variables into categorical variables by dividing them into groups based on their quartiles. The percentage of embryos that implanted for each timing quartile was calculated to assess the distribution of implantation in the different categories; Chi-square tests were used to compare between categorical data.

Results and Discussion

7- Time Lapse analysis

For this thesis work, we collected the data obtained from the morphokinetic evaluation of 369 embryos cultured in the Miri TL from about 97 women undergoing IVF cycles.

After micro-injection, inseminated oocytes were incubated under the previously described culture conditions and the development of the zygotes and embryos was assessed during the *in vitro* culture according to standard morphological evaluation parameters. 255 blastocysts were formed from cultured embryos and 97 were transferred with a 27% positive outcome. 77 blastocysts underwent PGT to assess chromosomal status and define the euploidy or aneuploidy status.

Morphokinetic parameter	Definition
tPNa	Time when both pronuclei are visible
tPNf	Time when both pronuclei had faded
t2- t9	Time of cleavage from two to nine blastomeres
tComp	Time of first evidence of compaction
tMor	Time to full embryo compaction (morula)
tEarlyB	Time to start of blastulation and first sign of a cavity in visible
tB	Time to formation of a full blastocyst, where blastocele completely fills the embryo and trophectoderm and inner cell mass are well defined
tExpB	Time to expanded blastocyst, where the maximum volume is reached and the zona pellucida begins to thin

Table 2: Definitions of the timings used for the evaluation of cellular events during embryo development.

In this study the data obtained from the evaluation of the timing of embryonic divisions were analyzed retrospectively; the sequence of events was recorded in hpi (hours post insemination) starting from fertilization through the appearance of the two pronuclei (tPNa) and their disappearance (tPNf), towards the following cellular divisions (time of cleavage from 2 to 9 cells: t2, t3, t4, t5, t6, t7, t8, and t9). The times were annotated at the first frame in which the blastomeres were seen as separated by individual membranes; tComp (time of compaction) is defined by the first evidence of compaction, while timing of morula (tMor), is defined as the stage at which it was no longer possible to visualize the individual blastomere membranes and full embryo compaction

was achieved. Timing of early blastocyst (tEarlyB) consist of the time from insemination to the beginning of blastulation and formation of the blastocoele; time to Blastocyst (tB) is instead defined by the formation of a ‘‘full blastocyst’’ when the blastocoele cavity filled the embryo and the inner cell mass and trophectoderm tissues were distinguishable from each other. The successive step was the time of expanded blastocyst (tExpB), which consist in the increase of the overall volume of the embryo and expansion of the blastocoele cavity; this is the time frame after the zona pellucida starts to thin, so that the blastocoel was as full as possible. The definition of variables used in the analysis are reported in Table 2.

Other characteristics associated with embryo quality (e.g. fragmentation or multinucleation) were not taken into consideration in this study in order to assess separately the specific impact of cleavage dynamics on subsequent development during the interval of interest. Data obtained through the record of cellular divisions parameters of cultured embryos were retrospectively analyzed in order to evaluate how this type of assessment can represent a contribution in IVF cycles and can guide the embryologist in the selection of best quality embryos; the outcomes chosen in this evaluation are those of greatest interest in IVF treatments: blastocyst development, implantation potential and the state of euploidy or aneuploidy of the embryonic genetic heritage.

7.1- Blastocyst development

The objective of this part of the study was to investigate how the morphokinetic evaluation of embryonic development can allow to identify parameters associated with blastocysts formation and which, therefore, can be used to select embryos with greater potential.

Since in this part of the study the cell divisions relative to embryos that have reached the blastocyst stage (n= 255) or not (n= 114) were evaluated, the timings analyzed are those between tPNa and t8; the average timing and standard deviations of these data are presented in Table 3.

Parameter	Blastocyst stage embryos		8-cell stage embryos	
	Mean (hpi)	SD (hpi)	Mean (hpi)	SD (hpi)
tPNa	8,18	2,04	8,62	2,41
tPnf	23,85	2,73	24,08	3,3
t2	26,68	2,94	27,30	3,63
t3	36,98	4,88	36,45	6,81
t4	39,57	4,76	41,17	6,49
t5	50,45	6,86	50,51	9,84
t6	53,81	7,3	55,64	10,63
t7	58,15	8,76	60,72	11,33
t8	63,63	10,21	66,52	13,65

Table 3: Annotated timings for blastocyst stage and cleavage stage embryos. Mean and standard deviation are reported for each parameter in hours post insemination (hpi).

From a first observation of the reported data, it is evident that most of the division timings show a faster development in embryos that have reached the blastocyst stage compared to those that have stopped at the cleavage stage. In particular, timings are initially undistinguishable between the two groups but from the 4-cell stage, mean values of t4, t6, t7 and t8 in the cleavage stage group are of 41,17 hpi, 55,64 hpi, 60,72 hpi and 66,52 hpi respectively, while in embryos that have reached the blastocyst stage these timings are reduced (t4: 39,57 hpi; t6: 53,81 hpi; t7: 58,15 hpi; t8: 63,63 hpi). This trend agrees with the Istanbul Consensus on Embryo Assessment (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011) and with what has been reported by numerous authors in literature, according to which a too slow development may indicate poor embryo quality (Edwards et al, 1980; Giorgetti et al, 1995; Ziebe et al, 1997; Van Royen et al, 1999); in fact, during preimplantation development, different cell cycle checkpoints are activated in response to DNA damage, leading to cell cycle delay or arrest. Therefore, human embryos that develop to each cleavage stage at an appropriate time might show high developmental competence. Progress to each cleavage stage after an adequate interval between successive divisions might be a prerequisite for normal development; thus, the observation of the early development of an individual embryo might be a powerful tool to select those with high developmental competence (Hashimoto et al, 2012).

The presence of a larger standard deviation in the group that did not reach the blastocyst stage also indicates greater variability in the times of division in embryos with less development potential.

To establish when this difference can be considered a valid parameter for distinguishing embryos according to their development potential, the median values of the cellular divisions were compared. The delay in cleavage times of embryos that did not develop to the blastocyst stage was found to be significant especially in the early stages of embryo development (Table 4), in particular for tPNf (23,57 hpi versus 24,86 hpi; *P*= 0,0004), t2 (26,27 hpi versus 28,17 hpi; *P* <0,0001) and t4 (38,96 hpi versus 41,02 hpi; *P*= 0,01) (Figure 9). A significant difference was also found for t7 (26,27 hpi versus 28,17 hpi; *P*= 0,04).

Parameter	Blastocyst stage embryos			8-cell stage embryos			*P-value*
	25th percentile (hpi)	Median (hpi)	75th percentile (hpi)	25th percentile (hpi)	Median (hpi)	75th percentile (hpi)	
tPNa	6,73	7,88	9,39	6,88	8,165	9,68	NS
tPnf	21,8	23,57	25,63	22,63	24,86	27,15	0,0004**
t2	24,43	26,27	28,37	26,11	28,17	30,71	<0,0001**
t3	34,54	37,03	40,1	30,38	36,54	42,09	NS
t4	36,45	38,96	42	36,87	41,02	44,88	0,01*
t5	46,76	50,81	53,86	42,85	50,13	57,23	NS
t6	49,7	52,98	57,49	49,1	55,17	62,42	NS
t7	52,6	55,97	62,38	52,58	58,97	66,83	0,04*
t8	55,72	61,84	70,54	55,49	64,05	74,91	NS

Table 4: Annotated timings for blastocyst stage and cleavage stage embryos. Values corresponding to the 25th percentile, median and 75th percentile are reported for each parameter in hours post insemination (hpi). **P* < 0,05; ***P* < 0,01. NS: not statistically significant.

Other works in the literature report similar findings (Wong et al, 2010; Hashimoto et al, 2012; Conaghan et al, 2013), suggesting that success or failure of embryo development is likely to be determined at least in part by inheritance of maternal transcripts, which we observed to be expressed at altered levels in abnormal embryos; during these stages, in fact, the Embryonic Genome Activation (EGA) has not yet occurred.

Comparison of the times between the five and eight blastomere stage, however, revealed no significant differences except for the seven-cell stage, where the median time is 55,97 hpi in those embryos that have reached the blastocyst stage versus 58,97 hpi (*P* <0,04).

According to what has been observed, we can use the data obtained from this retrospective evaluation to define new parameters that can help us to identify the embryos with the greatest potential during the early stages of development; in fact, through the time lapse evaluation it is possible to identify in a cohort of embryos of the same morphological grade, those with the fastest division times (in particular tPNf, t2 and t4) and which can be considered those with greater development potential and therefore be selected for transfer.

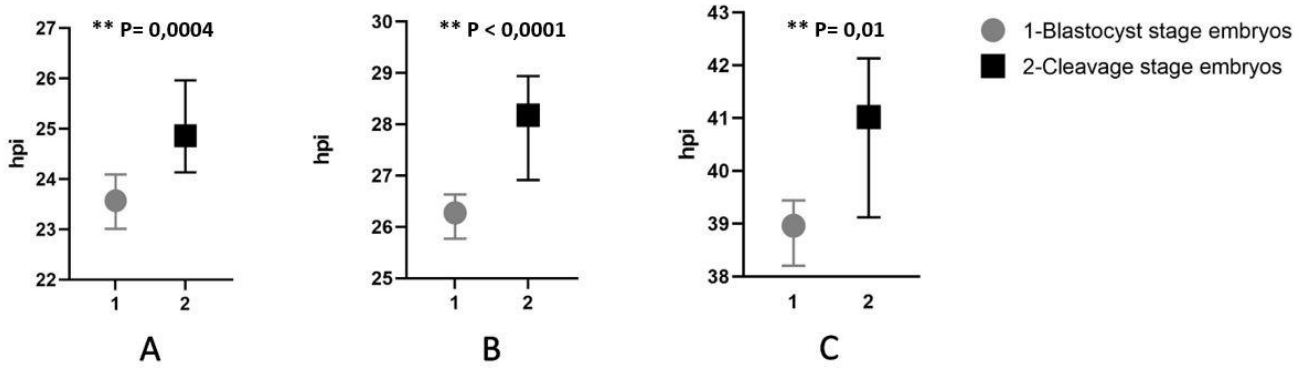

Figure 9: Distributions of cleavage times for Blastocyst stage embryos and Cleavage stage embryos. A: tPNf; B: t2; C: t4. Timings are plotted as median with 95% CI. hpi: Hours post insemination

As embryo developmental potential can be assessed through the analysis of cleavage kinetics, it may be feasible to translate these basic studies to clinical applications. Current morphological and growth criteria that are commonly used to assess embryo viability during cleavage stage may both underestimate and overestimate embryo potential (Racowsky, 2002), usually leading to the transfer, at this stage, of two embryos with consequences such as multiple births and miscarriages. Given the uncertainties associated with evaluation at day 3, some clinics have turned to longer culture to assess embryo potential (Milki et al, 2000; Gardner et al, 2004), as embryos transferred at the blastocyst stage have a higher implantation rate compared with embryos transferred at day 3. Moreover, in prolonged embryo culture some presumably non-viable embryos arrest their development facilitating the ultimate selection of the blastocyst for transfer. However, this practice involves prolonged in vitro culture and may increase the chance of altered gene expression and epigenetic inheritance. Thus, the possibility of being able to define embryo selection parameters during the early stages of development allows us to select single embryos with greater potential and

to be able to carry out the transfer on the third day of development and avoid all the risks related to a prolonged in vitro culture. However, the question of embryo-endometrial interaction should not be overlooked. In fact, embryo transfer at the blastocyst stage might still prevail as a more favourable strategy, as a result of a better temporal coordination between embryo invasiveness and endometrial receptivity (Dal Canto et al. 2012).

7.2- Implantation potential

For the evaluation of the implantation potential, the division times of 97 blastocysts transferred to the uterus were retrospectively analyzed; only the cases in which the number of transferred embryos was equivalent to the number of sacs (100% implantation, n= 26) in comparison to transfers that did not give rise to implantation (0% implantation, n= 71) were included in this analysis.
The average timing and standard deviation of the cellular division from pronuclear appearance (tPNa) to the expanded blastocyst stage (tExpB) for the embryos that have implanted and for those who have given a negative pregnancy outcome are presented in Table 5.

Parameter	Implanted embryos		Not-implanted embryos	
	Mean (hpi)	SD (hpi)	Mean (hpi)	SD (hpi)
tPNa	8	1,87	7,77	1,83
tPnf	23,31	2,53	23,5	2,57
t2	26,03	2,49	27,52	1,85
t3	37,49	3,27	38,47	3,57
t4	39,09	3,94	41,46	4,47
t5	50,72	5,35	53,40	5,74
t6	52,97	4,34	54,72	6,01
t7	55,19	5,12	58,07	8,70
t8	60,05	7,71	62,55	10,40
t9	71,41	6,54	71,76	9,43
tComp	79,42	7,78	83,07	9,30
tMor	91,92	6,45	94,82	8,83
tEarlyB	99,22	6,49	103,48	9,46
tB	106,07	6,68	110,98	10,81
tExpB	113,52	7,40	117,93	10,81

Table 5: Annotated timings for implanted and not implanted embryos. Mean and standard deviation are reported for each parameter in hours post insemination (hpi).

As was observed for embryos with greater development potential, also in this case the embryos that did not lead to pregnancy show longer division times for all the annotated parameters than those

that implanted, suggesting once again that slower development is associated to a reduced embryonic quality.

Since most of the exact timings of embryo events in the group of not-implanted embryos do not follow normal distributions (Shapiro-Wilk test), in order to understand if there is a difference between the data obtained from the annotation of the division times of the implanted and non-implanted embryos, the median values of these data were compared (Table 6).

Parameter	Implanted embryos			Not-implanted embryos			*P-value*
	25th percentile (hpi)	Median (hpi)	75th percentile (hpi)	25th percentile (hpi)	Median (hpi)	75th percentile (hpi)	
tPNa	6,63	7,79	9,08	6,64	7,6	9,08	NS
tPnf	21,54	22,63	24,72	21,51	23,01	25,11	NS
t2	24,46	25,3	26,74	26,31	27,46	28,37	0,0002**
t3	35,28	37,18	39,75	37,02	38,9	40,46	0,0338*
t4	36,24	37,6	41,43	38,82	41,01	44,06	0,0186*
t5	46,78	51,24	53,73	49,51	52,72	57,32	0,0324*
t6	49,78	52,5	55,57	49,26	52,69	57,46	NS
t7	51,34	53,725	58,53	52,58	55,84	61,01	NS
t8	53,84	58,6	66,29	54,32	60,56	68,52	NS
t9	68,17	69,84	74,16	65,37	70,08	77,08	NS
tComp	72,93	80,01	85,21	75,19	82,76	90,35	NS
tMor	87,28	91,3	96,68	87,83	94,93	100,06	NS
tEarlyB	94,33	98,77	104,99	96,5	102,67	110,15	0,04*
tB	101,32	104,66	110,35	102,38	109,15	117,82	NS
tExpB	109,42	111,19	117,63	109,52	117,09	123,9	NS

Table 6: annotated timings for implanted and not-implanted embryos. Values corresponding to the 25th percentile, median and 75th percentile are reported for each parameter in hours post insemination (hpi). *$P < 0,05$; **$P < 0,01$. NS: not statistically significant.

A significant difference can be observed between the data corresponding to the annotations relating to the first embryonic divisions and in particular for t2 (25,3 hpi versus 27,46 hpi; $P= 0,0002$), t3 (37,18 hpi versus 38,9 hpi; $P= 0,0338$), t4 (37,6 hpi versus 41,01 hpi; $P= 0,0186$) and t5 (51,24 hpi versus 52,72 hpi; $P= 0,0324$) However, no significant difference is observed in the subsequent divisions, except for the time taken by the embryos to form an early blastocyst, where the median

value for tEarlyB is 98,77 hpi for the implanted embryos and 102,67 hpi for the not-implanted embryos (*P*= 0,04).

The results obtained, therefore, seem to highlight the importance of the first embryonic divisions also as regards the implantation potential, allowing to identify morphokinetic parameters that can help the embryologist to distinguish the embryos that can implant and give pregnancy.

To this end, the timings corresponding to the division times of early developmental stages that were found to be significantly different between the two groups (t2 to t5) were divided according to their quartiles. The four quartiles for the timing of each of the investigated parameters are presented in Table 7, together with percentages of implanting embryos in each quartile.

Parameter	Q1		Q2		Q3		Q4	
	Limit (hpi)	Implantation (%)	Limit (hpi)	Implantation (%)	Limit (hpi)	Implantation (%)	Limit (hpi)	Implantation (%)
t2	**<25,52**	**58**	**25,52-27,07**	**31**	27,07-28,22	8	>28,22	11
t3	**<36,47**	**38**	**36,47-38,57**	**35**	38,57-40,2	15	>40,2	23
t4	**<37,63**	**46**	**37,63-40,76**	**31**	40,76-43,28	15	>43,28	8
t5	**<48,84**	**31**	**48,84-52,36**	**35**	52,36-55,92	27	>55,92	8

Table 7: Timing of t2, t3, t4 and t5 grouped in quartiles (Q1, Q2, Q3 and Q4) from 97 transferred embryos. Numbers in bold indicate the two quartiles with the highest implantation percentages.

The categories defined by these quartiles were used to establish optimal ranges based on the two consecutive quartiles with highest implantation probabilities: <27,07 hpi for t2, <38,57 hpi for t3, <40,76 hpi for t4 and <52,36 hpi for t5. For all cleavage times assessed (t2, t3, t4 and t5), embryos whose cleavage were completed in the two first quartiles displayed the highest implantation rates, and were consequently combined in an optimal range for each parameter (Fig. 10).

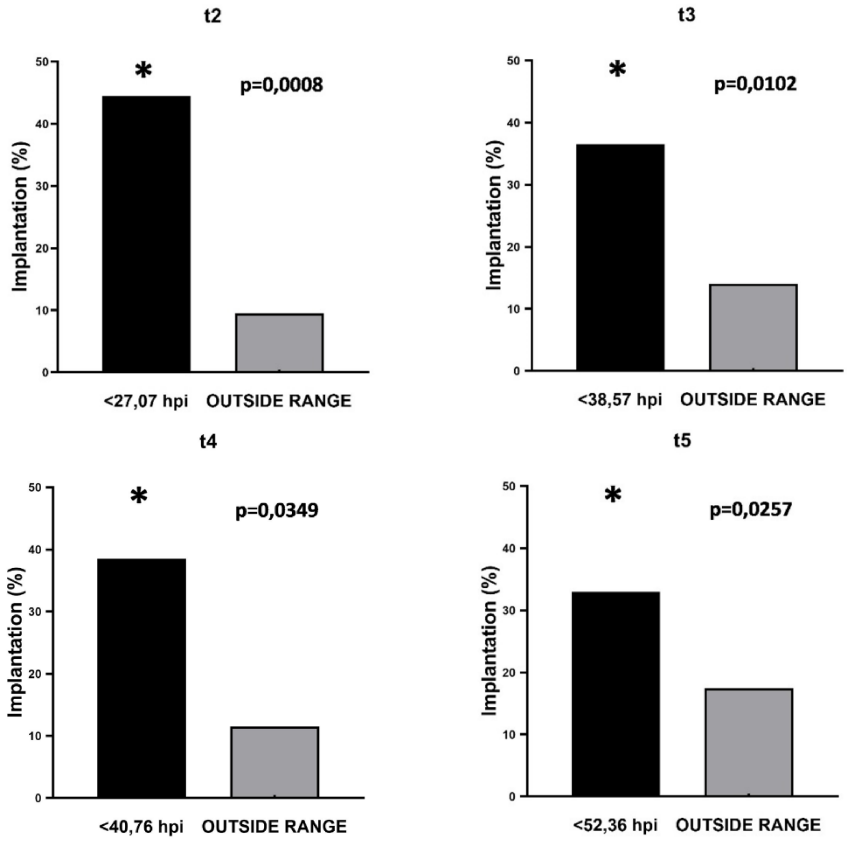

Figure 10: Percentage of implanting embryos with cell division times inside or outside ranges defined by quartile limits for the total data set. The four panels show ranges and implantation for division to 2 cells (t2), division to 3 cells (t3), division to 4 cells (t4) and division to 5 cells (t5).

As can be seen from the reported data, there is a significant difference in implantation rate between embryos within the optimal range as opposed to those outside the range for all the considered cleavage times.

For t2, the difference in implantation rate is 35%, for t3 22%, for t4 we found 27% difference and for t5 it amounts to 15%; the significance of these results therefore allows us to consider these parameters as good indicators of the implantation potential of the embryos and to be able to use this marker to identify those to be transferred. Current data confirm the importance of cleavage kinetics as a strong predictor of implantation potential also affirmed in the study by Meseguer and colleagues (Meseguer et al, 2011), in which they found that are the first embryonic divisions (in particular during the 5-cell stage) to be the most predictive of implantation ability.

There are many plausible explanations for the observed association that could be directly or indirectly related to cellular processes implicated in cell division. Observed variations in the timing

of embryonic development may be related to culture conditions that can affect embryo metabolism coupled with intrinsic factors within the oocyte, such as ooplasm maturity (Escrich et al, 2010) and/or sperm (paternal effect may affect the duration of synthesis phase). Furthermore, chromosomal abnormalities may delay DNA replication (Lechniak et al, 2008), thus altering the length of cell cycles and divisions. Our data revealed developmental dynamics for transferred embryos related to clinical implantation potential, suggesting that there is an optimal time range for parameters characterizing the early embryonic cell divisions: embryos that cleave at intermediate time points have significantly improved chance of implantation when compared with embryos that developed slower.

Our results demonstrates that routine time-lapse monitoring of embryo development in a clinical setting, provides more information about developmental parameters that differ between implanting and non-implanting embryos. The studies in the literature, however, show conflicting results regarding the possibility of using TLT to select the embryos with the greatest implantation potential. For example, in the work by Goodman (Goodman et al, 2016) is reported a not significant increase in the clinical pregnancy rate when the embryos were selected based on morphokinetics versus standard morphological assessment. The study by Rubio and colleagues (Rubio et al, 2014), instead, confirms that morphokinetic assessment can improve embryo selection, but they concluded that morphokinetic variables allow us to reject embryos with lower implantation while distinguishing those with higher implantation probabilities.

7.3- Euploidy or Aneuploidy status

It is widely accepted that a major cause of failed implantations or miscarriage following embryo transfer is the unknowing transfer of aneuploid embryos. Several studies indicate that aneuploidy is very common in human gametes and embryos and it is therefore one of the major causes of implantation and pregnancy failures (Fragouli and Wells, 2011; Munne et al, 2009; Swain, 2013). Although the intrinsic mechanisms on how such errors are generated are largely unknown, errors in meiosis during gametogenesis and mitosis during the course of preimplantation embryo development create chromosomal imbalances which impair implantation and early embryonic development. Many of these errors do not create distinctive differences during static embryo scoring process and, therefore, chromosomally altered embryos cannot be distinguished from euploid, leading to treatment failures (Fragouli et al, 2014; Munne et al, 2009). To date, the only

way available for assessing ploidy in embryos has been polar body, blastomere or trophectoderm biopsy with Preimplantation Genetic Testing (Campbell et al, 2013).

The evaluation of embryonic development through the Time Lapse System was also carried out on a group of 77 blastocysts that underwent trophectoderm biopsy and Preimplantation Genetic Test (PGT), with the aim of evaluating the presence of differences in cleavage times between euploid (normal chromosomal complement; n= 40) and aneuploid (presence of monosomy or trisomy; n= 37) embryos. Since the trophectoderm biopsy is performed on blastocysts that have reached the expansion state, the timing evaluation was performed for all cellular events starting from tPNa, up to tExpB; the average timing and standard deviations of these data are presented in Table 8.

Parameter	Euploid embryos		Aneuploid embryos	
	Mean (hpi)	SD (hpi)	Mean (hpi)	SD (hpi)
tPNa	8,08	2,78	8,54	1,74
tPnf	23,30	2,39	24,63	2,47
t2	26,06	2,39	27,59	2,71
t3	36,54	4,1	38,55	3,82
t4	39,03	4,11	40,5	4,56
t5	50,1	5,72	52,93	7,53
t6	52,97	5,92	56,69	7,41
t7	57,36	7,69	59,51	6,69
t8	62,14	8,49	65,65	9,89
t9	71,95	7,83	76,06	8,65
tComp	83,48	7,96	85,16	9,71
tMor	94,09	9,27	97,5	9,82
tEarlyB	103,82	9,42	107,15	9,35
tB	111,18	10,14	116,06	11,3
tExpB	120,78	10,81	126,21	10,39

Table 8: annotated timings for euploid and aneuploid embryos. Mean and standard deviation are reported for each parameter in hours post insemination (hpi).

From the retrospective analysis of the data, we can observe that, on average, the times of division of embryos that have presented an altered chromosomal status are longer in time compared to euploid embryos; this confirms what was observed in the groups previously analyzed, since the embryos with less development and implantation potential showed longer cleavage times. However, the standard deviation appears to be similar in the two groups, indicating a lower timing variability even in the aneuploid embryo group (Table 8).

To determine which parameters may be correlated with ploidy the significance of the differences between the analyzed groups was evaluated by comparing the median values (Table 9).

Parameter	Euploid embryos			Aneuploid embryos			*P-value*
	25th percentile (hpi)	Median (hpi)	75th percentile (hpi)	25th percentile (hpi)	Median (hpi)	75th percentile (hpi)	
tPNa	6,4375	7,75	9,41	7,045	8,48	9,915	NS
tPnf	21,46	23,04	24,88	22,66	24,88	26,36	0,01*
t2	24,43	25,67	28,04	25,71	27,67	29,34	0,01*
t3	34,74	36,24	38,89	35,59	38,93	40,94	0,04*
t4	36,27	38,08	41,04	36,94	40,3	42,86	NS
t5	46,9	49,65	53,33	49,67	52,95	55,33	0,04*
t6	49,65	51,77	57,19	51,1	54,12	58,7	NS
t7	51,24	55,29	61,93	52,67	56,61	66,28	NS
t8	56,36	62,33	67,39	56,91	62,73	72,84	NS
t9	66,4	71,98	77,52	71,66	74,36	80,2	NS
tComp	78,745	82,94	88,35	78	84,07	91,57	NS
tMor	88,16	92,85	99,82	91,86	97,55	102,84	NS
tEarlyB	96,615	104,41	110,13	100,13	107,31	113,22	NS
tB	104,86	109,64	116,91	107,77	115,07	122,74	0,04*
tExpB	110,82	121,03	130,79	118,32	127	133,47	0,03*

Table 9: Annotated timings for euploid and aneuploid embryos. Values corresponding to the 25th percentile, median and 75th percentile are reported for each parameter in hours post insemination (hpi). *$P < 0,05$. NS: not statistically significant.

The first significant parameter is the time of pronuclear fading, which was longer in aneuploid embryos compared with euploid embryos (24,88 hpi versus 23,04 hpi; $P= 0,01$). As Table X demonstrates, even the time of subsequent divisions to two, three and five blastomeres are statistically different between aneuploid and euploid embryos. For the subsequent divisions, however, no significant difference is observed despite cleavage times of aneuploid embryos remain constantly longer throughout development.

The comparison between the division times of euploid and aneuploid embryos it's again significant at the blastocyst stage ($P= 0,04$); in fact, tB has a median value of 109,64 hpi in chromosomally normal embryos compared to embryos with altered ploidy (tB= 115,07 hpi). This difference between the two groups of observed embryos continues even to the next stage of expanded blastocyst, where the median of tExpB for euploid embryos is 121,03 hpi and 127 hpi for aneuploid embryos, with a delay of about six hours to reach the stage necessary to perform the trophectoderm biopsy. From what emerges from our results, therefore, these division timings can be considered morphokinetic parameters associated with aneuploidy.

Interestingly, the differences were found to be significant at different stages of preimplantation development: in the human embryo only a subset of genes is activated before the 8-cell stage and the first hours of development rely on mRNA inherited from the gametes for survival and the up-regulation of embryonic genes starts from the 8-cell stage embryo (Xue et al, 2013; Zhang et al, 2009).

Although the cause of a temporal delay in aneuploid embryos compared with their euploid counterparts is not yet fully explained, there exist error detection and repair systems within the cell to prevent aneuploidy (Nasmyth and Haering, 2009). It is highly probable, therefore, that mitotic errors in individual cells at this stage of the rapidly developing embryo involve complex biochemical systems delaying karyo- and cytokinesis, which result in the observation of delayed blastulation.

The studies in the literature shows different and conflicting results about the correlation between chromosomal status and morphokinetic parameters.

Alfarawati et al. (2011) previously compared blastocyst qualitative morphology with ploidy and demonstrated only a weak association between blastocyst morphology and aneuploidy. They reported that, concerning the growth rate of blastocysts, there was an insignificant trend toward aneuploid embryos showing slower progression to the most advanced blastocyst stages and that embryos with aneuploidy were most delayed.

One of the main works on the correlation between morphokinetic parameters and aneuploidy status is the one conducted by Campbell and colleagues (Campbell et al, 2013); this study indicates that initiation and completion of blastulation appears to be the most reliable non-invasive method of ranking the risk of aneuploidy in advanced-stage embryos. However, it isn't as absolute as gaining accurate chromosome copy number from biopsied cells, but it is an important tool to enhance the chances of a live birth following IVF by non-invasive means. They concluded that, with time-lapse monitoring of embryo development to blastocyst in a closed incubation system, it is possible, where there are alternative embryos available within a cohort, to avoid the selection of embryos having a high risk of aneuploidy and to preferentially select embryos with a greatly reduced risk of aneuploidy based on morphokinetic timing.

Basile et al. also correlated morphokinetics with embryo euploidy on patients undergoing PGT. In this case, the model identified the variables t5-t2 as the most relevant variables related to a normal chromosomal content. Once again, they concluded that this correlation could aid the embryologist in selecting embryos with higher probabilities of being euploid and this represents an excellent tool; however, it should be acknowledged that it is not possible to guarantee chromosomal normality

through this approach. In contrast, in the work by Vera-Rodriguez (Vera-Rodriguez et al, 2015) only two parameters were found to be statistically different between both groups: the time between pronuclear disappearance and the start of the first cytokinesis. The correlation between morphokinetic parameters up to the blastocyst stage and aneuploidy diagnosed by trophectoderm biopsy and comprehensive chromosomal screening, therefore, could not be confirmed.

8- Presence of EV in the embryo-spent media: preliminary data from an ongoing analysis.

Extracellular vesicles and exosomes have attracted significant attention in the reproduction field due to their various roles from gametogenesis to embryo development and implantation. Although EVs have not been as intensively studied during preimplantation embryo development as in other cell types, it seems that their characteristics (mean size, mode of size and particle concentration, as well as their cargo) can be associated with embryo stage and quality embryos (Ferreira *et al.* 2013, Saadeldin *et al.* 2014, Mellisho *et al*. 2017).

For these reasons, we started a preliminary analysis on the spent culture media of the embryos cultured *in vitro* with the aim of evaluating the presence and quantity of extracellular vesicles (EVs) that can be considered non-invasive biomarkers able to provide additional information on embryo quality.

In order to evaluate the presence of EVs in the embryo culture media, the Nanoparticle Tracking Analysis (NTA) was performed on spent media collected at the end of the embryo culture of 21 blastocysts incubated in the Miri Time Lapse system. PBS solution and blank culture media subjected to the same incubation conditions were included as negative control.

Size distributions and total concentration of EV-like particles by Nanoparticle tracking analysis (NTA) is a light-scattering technique widely used to assess size and concentration of particles population. EVs are suspended in a buffer solution and irradiated by a laser; some light will be scattered and dispersed, thus this scattered light is captured by a microscope, and NTA software tracks the movement of each particle returning back results. We made and measured diverse dilutions of the same sample as to assure the linearity. For this analysis, we have selected the camera level (16) and the threshold of Detection (D) that provides correct sizing of near-to 100 nm peaks. Before all sample measurements, we confirmed that the PBS diluent contained less than 1.0 particle per frame by measuring the PBS for 60 s in static and flow mode. Total (TCT) and valid (TVT) particle tracks are collected, and the measurements flagged as reliable if their ratio was less than 5. As to evaluate the size both the mean size and mode size are measured.

Sample	P/ml	P/ml *dl	P/frame	Size (mean)	Size (mode)	TCT/TVT
1	2,0E+08	1,0E+12	10,4	86,0	76,0	2,70
2	8,0E+08	4,0E+11	40,4	117,0	107,0	3,5
3	4,1E+08	4,1E+11	21,1	87,0	80,0	3,2
4	1,5E+09	1,5E+12	75,4	89,0	64,0	4,0
5	4,5E+08	4,5E+12	23,0	95,0	90,0	3,1
6	4,3E+08	2,2E+12	21,9	88,0	71,0	2,7
7	4,1E+08	4,1E+11	20,8	106,0	82,0	4,8
8	5,7E+08	5,7E+12	29,0	90,0	77,0	2,7
9	4,9E+08	2,5E+12	24,8	102,0	79,0	2,8
10	7,9E+08	7,9E+11	40,0	98,0	74,0	2,9
11	9,3E+08	4,7E+11	47,3	106,0	101,0	3,0
12	1,7E+09	1,7E+12	85,8	94,0	71,0	3,1
13	1,2E+09	1,2E+12	61,1	95,0	82,0	3,2
14	1,1E+09	1,1E+12	55,7	81,0	69,0	3,1
15	5,5E+08	5,5E+12	27,9	106,0	84,0	3,1
16	1,3E+09	1,3E+12	66,3	108,0	101,0	3,1
17	1,3E+09	1,3E+12	64,3	82,0	70,0	2,9
18	5,7E+08	2,9E+12	28,9	103,0	81,0	3,1
19	1,2E+09	1,2E+13	61,5	69,0	58,0	2,7
20	5,6E+08	5,6E+11	28,6	79,0	59,0	2,9
21	6,6E+08	6,6E+11	33,5	113,0	105,0	3,8

Table 10: Size and concentration of EVs populations measured with Nanoparticle tracking analysis. P/ml: particles/ml; P/ml *dl: particles/ml X dilution factor; P/frame: particles/frame; Size (nm); TCT: Total particle tracks; TVT: valid particle tracks.

The data obtained from this analysis are shown in Table 10. EVs population from cultured embryos show variability for all the analyzed parameters; in particular, the number of particles/ml (multiplied by the dilution factor) ranges from a minimum of 4.00E + 11 to a maximum of 1.20E + 13. Since the concentration of extracellular vesicles in the embryo culture medium is a parameter that can be easily and quickly measured using NTA, we evaluated whether this analysis can be compared with the data obtained from the annotation of the division timing of the same embryos during the in vitro culture. To this end, the values corresponding to the number of particles/ml (multiplied by the dilution factor) were divided according to their quartiles and associated with the timing (from tPNa to tExpB) of cellular divisions.

Parameter	Q1 (<6,1E+11)	Q2 (6,1E+11-1,3E+12)	Q3 (1,3E+12-2,7E+12)	Q4 (>2,7E+12)
	mean (hpi)	mean (hpi)	mean (hpi)	mean (hpi)
tPNa	8,20	8,27	8,04	6,98
tPnf	27,02	25,97	25,44	22,91
t2	29,81	28,68	27,75	25,58
t3	42,20	40,54	40,86	35,61
t4	43,25	42,36	41,47	37,49
t5	58,04	56,87	55,29	48,75
t6	60,30	58,93	57,36	51,05
t7	64,94	59,66	58,55	53,61
t8	69,13	62,52	61,88	58,37
t9	82,17	76,78	76,83	70,20
tComp	88,23	88,72	87,10	82,03
tMor	99,96	98,80	95,66	90,68
tEarlyB	109,62	108,60	104,46	101,11
tB	118,93	116,92	112,18	109,62
tExpB	127,92	125,08	123,63	120,96

Table 11: Timing of cellular events divided according to quartiles of the number of particles/ml* dilution factor detected in the spent media from 21 embryos.

From the obtained results (Table 11), we can observe that the embryos whose culture medium analysis shows a lower concentration of extracellular vesicles appear to be those with slower divisions for all the annotated parameters. In particular, in the cellular divisions ranging from t5, this difference seems to be more evident; while the mean value of t5 for embryos in which the number of particles/ml is < 6,1E+11 corresponds to 58,04 hpi, in the group where this range is greater than 2,7E+12 the time taken to reach 5 blastomeres is 48,75 hpi, with a difference of about 10 hours. This trend is also observed in subsequent divisions up to time to 9 cells, in which the t9 is 82,17 hpi in the embryos corresponding to Q1, and 70,20 hpi in those with a higher concentration of particles. From compaction to the expanded blastocyst stage, these values are less variable in the different groups. although the same trend continues to occur.

According to what was reported in the results presented previously on the evaluation of the timing of embryo development, slower embryo division times are correlated to lower development and implantation potential (Alpha Scientists and ESHRE Special Interest Group of Embryology, 2011), therefore, the results obtained about concentration of extracellular vesicles, seem to be able to correlate with the morphokinetic parameters, indicating that a greater quantity of EVs could indicate embryos of lower quality and development potential. However, due to the small number of cases

examined and the limit due to the limited amount of material available, it is not possible to establish the significance of this association.

Since EVs are recognized as carriers of proteins, lipids, and nucleic acids and can transfer this cargo to recipient cells, participating in cell-to-cell communication (Raposo and Stoorvogel 2013; Kowal et al, 2014; Battaglia et al, 2019), in recent years several studies have focused on how they can provide information on embryo viability and can be used as non-invasive markers of embryonic quality but so far with non-convincing results, and the predictive value of proposed systems is still controversial and unfeasible (Capalbo et al. 2016).

Future studies aim to characterize these vesicles and their molecular content in order to obtain more precise information on the quality of the embryos from which they are produced, in order to be able to identify new biomarkers to be introduced into clinical practice

CONCLUSIONS

In recent years, the use of Time Lapse Microscopy for the study of early embryo development has attracted considerable interest in the field of clinical embryology, as this technology has offered a new range of opportunities to study cell cleavage during early human development and select embryos with higher developmental potential; in fact, as embryo development is a dynamic process, it would not be possible to assess the presence of some cellular events only with the use of the standard morphological assessment.

In this work, data obtained through the record of cellular divisions timings of embryos incubated in a Time Lapse System were retrospectively analyzed in order to identify parameters that can be used as markers able to provide more information on embryo quality and can contribute to the success of IVF treatments. Our results confirm the importance of cleavage kinetics as a strong predictor of embryo development and implantation. In particular, this study observed that embryos arresting during the cleavage stages develop more slowly than embryos compared to embryos that reach the blastocyst stage. Since single embryo transfer is considered the best practice for the majority of patients undergoing IVF treatments, the possibility of being able to define embryo selection parameters during the early stages of development allows us to select single embryos with greater potential and to be able to carry out the transfer on the third day of development and avoid all the risks related to a prolonged in vitro culture. At the same time, we observed how some morphokinetic parameters can be correlated to implantation potential of the embryos, allowing us to identify the best embryos for transfer during the early stages of *in vitro* culture.

In conclusion, Time lapse technology can provide useful information that can be used in clinical practice; moreover, one of the main potential benefits of TLM systems is to provide undisturbed culture environment and minimize the undesirable risks for developing embryos since a highly controlled environment is crucial for a successful preimplantation embryo development.

The lack of specifically developed algorithms provoked the question if it would ever be possible to find a universal morphokinetic algorithm, which will work across different clinics and independent of conditions such as oxygen and/or insemination technique; moreover, constructing a universal algorithm requires a large dataset of embryos. The search for objective robust biomarkers of embryo viability continues, although to date, and despite significant research effort, no single reliable biomarker with a sufficiently high predictability of live birth has yet been identified: this may reflect the complexity of preimplantation development. Consequently, the search for biomarkers must no longer occur in isolation but in combination with other methodologies capable of investigating the physiology of the embryo.

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