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XXXII CYCLE

Novel strategies to improve human embryo selection in IVF: from morphokinetic analysis to Artificial Intelligence

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Abstract

Aim of the present thesis was to evaluate whether the dynamic observation of embryo development using Time-Lapse Technology (TLT) and the application of innovative computer-based approaches of Artificial Intelligence (AI) may represent novel strategies to optimize early embryo selection and improve pregnancy chance in modern In Vitro Fertilization (IVF). At first, we observed that morphokinetic analysis of the developing embryo coupled to conventional morphology may be useful in improving the embryo selection when embryo transfer is scheduled on day 3, as we obtained clinical outcomes comparable to the transfer of a single blastocyst on day 5. However, in the last decades the transfer at the blastocyst stage represents the gold standard in IVF aiming at maximizing the pregnancy chance and reducing the twinning risk. Thus, in a second study, we observed that embryo morphokinetics in the first 3 days of in vitro growth together to the expression of oocyte quality genes in the corresponding cumulus cells (CCs), were able to predict whether an embryo would timely reach the expanded blastocyst stage or would prematurely undergo developmental arrest. Our data opened the possibility of developing integrated prediction models and algorithms for early embryo selection at the cleavage stage including both morphokinetic and other embryological parameters. The extremely rapid increase in the quantity of data obtained in the last years faced the need to develop complex predictive algorithms to improve clinical outcomes in modern medicine. AI and Machine Learning (ML), in particular, represent intriguing strategies to build algorithms for features selection and objective classification with the aim of accurately predict a new event based on previously acquired experience. In our last research, we developed a ML algorithm including seven morphological and morphokinetic variables able to correctly identify embryo with the best chance to progress to the expanded blastocyst stage with an accuracy of 73%. These preliminary results suggested the possibility to improve the current strategy with the development of hierarchical models for a more accurate embryo classification. In the future, the introduction of ML approaches in the IVF labs for the optimization of embryo selection may represents a revolutionary scenario able to dramatically change the decision-making process and the personalization of the IVF treatments.

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1. Introduction

1.1 Assisted Reproductive Technology (ART)

1.1.1 Epidemiology and causes of human infertility

Infertility has been defined as a pathology by the World Health Organization (WHO), consisting in the failure to conceive after one year of unprotected intercourse. This reference time period has been established from the evidence that the pregnancy chance is around 25% per month in young couples so that the cumulative pregnancy chance results around 90% per year in most cases. This condition is further distinguished into *primary infertility*, referred to couples who have never become pregnant during one year, and *secondary infertility*, referred to couples who have been able to get pregnant at least once, but now are unable. Differently, *sterility* has been defined as the condition of one or both partners to be permanently unable to conceive, irrespective of the diagnostic work-up. It has been estimated that infertility affects approximately 15% of couples of reproductive ages, corresponding to 80 million couples worldwide, and over 45,000 new cases in Italy every year with a general increasing trend in the last twenty years. According to this phenomenon, it was observed an increase in the number of IVF (In Vitro Fertilization) centres to treat human infertility in Europe from 1997 to 2015 (De Geyter et al., 2020) (Figure 1).

Year	countries	clinics	Cycles	Cycle increase (%)	Infants born
1997	18	482	203 225		35 314
1998	18	521	232 225	+14.3	21 433
1999	21	537	249 624	+7.5	26 212
2000	22	569	275 187	+10.2	17 887
2001	23	579	289 690	+5.3	24 963
2002	25	631	324 238	+11.9	24 283
2003	28	725	365 103	+12.6	68 931
2004	29	785	367 056	+0.5	67 973
2005	30	923	419 037	+14.2	72 184
2006	32	998	458 759	+9.5	87 705
2007	33	1029	493 420	+7.7	96 690
2008	36	1051	532 260	+7.9	107 383
2009	34	1005	537 463	+1.0	109 239
2010	31	991	550 296	+2.4	120 676
2011	33	1314	609 973	+11.3	134 106
2012	34	1354	640 144	+4.9	143 844
2013	38	1169	686 271	+7,2	149 466
2014	39	1279	776 556	+13,1	170 163
2015	38	1343	849 811	+10.2	187 542
total			8 854 745		1 665 994

Figure 1. Number of institutions offering ART services, treatment cycles and infants born after ART in Europe, 1997–2015.

The increased trend in infertility is the consequence of profound changes in the socio-economic context occurred in the last twenty years. One of the critical factors in infertility is represented by the tendency of postponing the first pregnancy, mainly due to new working possibilities acquired by women, leading to a dramatic increase of age in women seeking a pregnancy. In addition, a deregulated lifestyle, characterized by the abuse of alcohol, drugs, food, smoking cigarettes, and the exposition to air pollutants acting as endocrine disruptors may also contribute to human infertility (Craig et al., 2017; Broughton et al., 2017; Mínguez-Alarcón et al., 2018; Bonde et al., 2016; Caserta et al., 2011). Infertility can affect both partners so that it is possible to distinguish *male factor* and *female factor*, to describe the infertility of male and female origin, respectively. According to the 2016 update of the Italian Registry of ART, the national distribution for causes of infertility among men and women is the following: male infertility, 29.3%; female infertility, 37.1%; combined infertility, 17.6%; unexplained infertility, 15.1%; genetic infertility, 0.9% (www.iss/rpma.it).

Unexplained infertility, also defined as *idiopathic*, is referred to couples whose cause of failed conception remains unknown even after an infertility work-up, usually including semen analysis in the man and assessment of ovulation and fallopian tubes in the woman.

The most common female factors of infertility are represented by age, endocrine disorders, poor ovarian reserve, endometriosis and altered tubal patency.

1) <u>Age</u>: the increased frequency in the unbalanced transmission of chromosomes in oocytes and early preimplantation embryos may cause aneuploidy, which is a major cause of infertility and implantation failure. Around 20% of human oocytes are estimated to be aneuploid and this increases exponentially from 30 to 35 years, reaching on average 80% by 42 years (Capalbo et al., 2017). In addition, advanced maternal age (AMA, ≥35) is also a risk factor for pregnancy loss, foetal anomalies, stillbirth, and obstetric complications, such as preeclampsia, hypertension and diabetes (Sauer et al., 2015).
2) <u>Endocrine disorders</u>: the presence of an altered hormonal profile for Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Oestradiol (E2) may affect the ovulatory process, leading to a condition of total or partial anovulation. Polycystic Ovary Syndrome (PCOS) represents the most frequent anovulatory condition among women undergoing assisted reproduction (Costello et al., 2019).
3) <u>Poor ovarian reserve</u>: follicular reserve dramatically decreases during the reproductive age, from puberty to menopause. The use of ovarian reserve markers, such as Antral Follicle Count (AFC), FSH and Anti-Mullerian Hormone (AMH), may provide useful instruments to predict pregnancy chance for spontaneous conception. They also represent the starting point for the individualization of controlled ovarian stimulation (COS) in IVF in order to offer the best treatment

tailored to the patient characteristics (La Marca et al., 2013). Furthermore, it has been observed that the strong relationship between the number of retrieved eggs and live birth rates, suggests that the number of eggs may be considered a robust surrogate outcome for clinical success in IVF (Sunkara et al., 2011).

4) Endometriosis: is a chronic and oestrogen-dependent disease characterized by the presence of ectopic endometrial tissue, comprising glands and stroma, outside the uterine cavity. There is a risk of infertility of 30%-50% (ASRM, 2012) due to adhesions on the peritoneal wall or the presence of ovarian endometriotic cysts that may promote anatomical distortion and the release of detrimental factors to oocytes, such as iron, reactive oxygen species, proteolytic enzymes and inflammatory molecules (Sanchez et al. 2014).
5) Tubal patency: fallopian tubes are physiologically involved in the transportation of the fertilized oocyte from the ovaries to the uterus where blastocyst may implant on day 5-6 of development. Bacterial infections by Chlamydia Trachomatis represent the most common cause of tubal inflammation, responsible of the Pelvic Inflammatory Disease (PID) and tube obstruction. It may lead to infertility or ectopic pregnancy (Budrys et al., 2012).

Other female causes of infertility may be associated to adenomyosis, congenital anomalies, miomas, polyps and Asherman Syndrome (AS), or to genetic diseases due to chromosomal alterations (e.g. Turner Syndrome).

The most common male factors of infertility are represented by varicocele, infections, cryptorchidism, obstructions and genetic diseases.

1) Varicocele: is the abnormal enlargement of the pampiniform venous plexus, which drains blood from the testicles back to the heart. It is characterized by an increase in

the scrotal temperature and the induction of a hypoxic state causing functional damage to the sperm. Varicocele occurs in around 15% to 20% of all men (Cavijo et al., 2017).

2) Infections: bacterial infections by Chlamydia Trachomatis, Mycoplasma or Ureaplasma may involve epididymis, prostate or seminal vesicles. The presence of a proinflammatory microenvirment may induce functional damage to the sperm and the formation of antibodies anti-spermatozoa. In addition, also HPV infection has been correlated to male infertility due to sperm binding by viral particles (Rintala et al., 2004).
3) Cryptorchidism: is the absence of one or both testes from the scrotum and represents the most common birth defect of the male genital tract. About 3% of full-term and 30% of premature infant boys are born with at least one undescended testis. However, about 80% of cryptorchid testes descend by the first year of life (the majority within three months), making the overall incidence of cryptorchidism around 1%. It is the most common cause of non-obstructive azoospermia and cancer (Docampo et al., 2015).
4) Obstructions: they may be the result of inflammatory processes, of congenital origin or iatrogenic. When congenital, the obstruction is bilateral and associated to azoospermia in presence of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene mutation (Chen et al., 2012).
5) Genetic diseases: represent the 15% of infertile men and are often associated to alterations of sexual chromosomes such as Klinefelter Syndrome (47, XXY), 47 XYY, microdeletions of Y chromosome. In addition, infertile men may carry monogenic mutations on CFTR, Androgen Receptor (AR) Insulin Like 3 (INSL3) and its receptor (RXFP2).

Based on the data provided by the Italian Registry of ART, the distribution of couples undergoing Assisted Reproduction Technologies (ART) in 2016 is shown in Figure 2 according to the infertility cause.

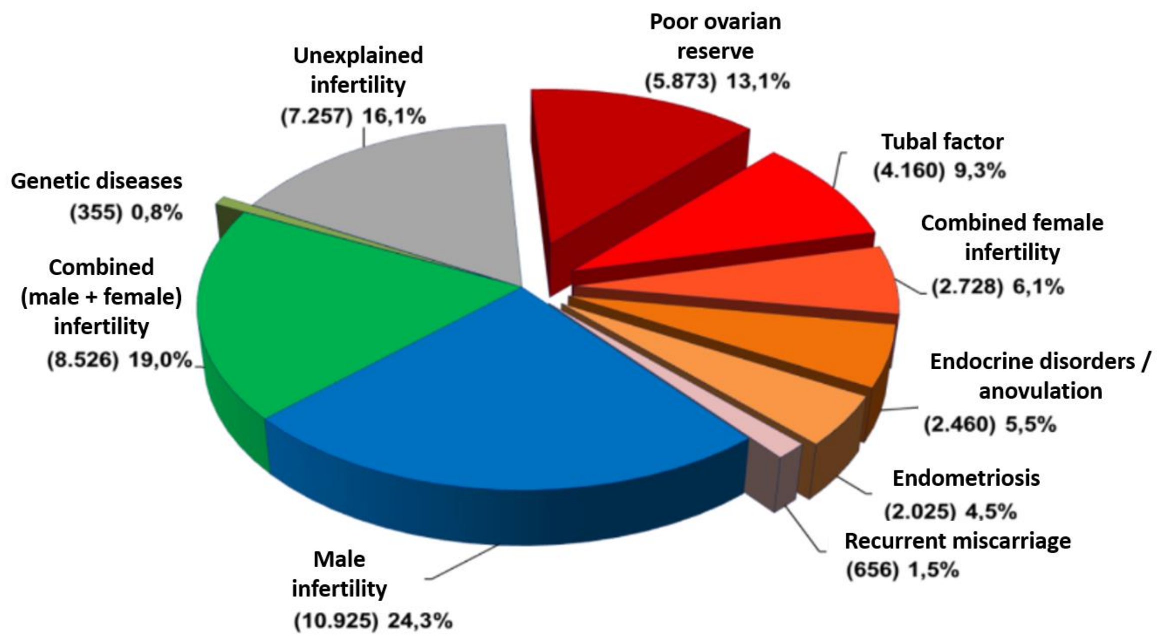

Figure 2. Distribution of couples undergoing Assisted Reproduction Technologies according to the infertility cause (modified from the Italian Registry of ART).

1.1.2 Treatment of infertility

Overall, the introduction of Assisted Reproductive Technologies (ART) to treat infertility in couples unable to conceive has led to a total amount of 1.5% of all infants born in the United States (Sunderam et al., 2015). According to cause of infertility (male, female or combined) different therapeutic approaches may be considered to achieve a successful pregnancy.

Intrauterine insemination (IUI)

Despite the fact that it has not been classified as a properly assisted reproductive technique (Zegers-Hochschild et al., 2006), it is currently used for a broad range of infertility indications, such as moderate male factor and unexplained infertility.

This technique is based on sperm preparation, mono-follicular growth monitoring by ultrasound and final ovulation induction using human Chorionic Gonadotrophin (hCG). A mild ovarian stimulation can also be coupled using clomiphene citrate (CC) or gonadotrophins (The ESHRE Capri Workshop Group, 2009). Capacitated sperm is then injected into the uterus 36 hrs after ovulation induction using a soft catheter. According to the data provided by the last registry of ESHRE, the delivery rates (DRs) per cycle after IUI resulted of 8.9% with homologous sperm (H) and of 12.4% after IUI with donor sperm (D). Twin and triplet DRs after IUI-H were 8.8% and 0.3%, respectively and 7.7% and 0.4% after IUI-D (European IVF-monitoring Consortium (EIM), 2020).

In Vitro Fertilization (IVF)

The in vitro fertilization is the process of fertilization where the female gamete, the egg, and the male one, the sperm, are combined outside the body in the artificial environment of a laboratory (in vitro). IVF was developed as a tool to overcome infertility due to problems with the fallopian tubes, anovulation, severe male factor and other conditions of unexplained

infertility. However, around 35-40% of all couples undergoing IVF treatments will not achieve pregnancy, suggesting that currently we still have a limited knowledge of the key factors affecting success, such as gamete and embryo quality and endometrial receptivity.

Louise Brown was the first child successfully born after her mother received a natural-cycle IVF treatment in 1978. In this case no ovarian stimulation was performed to enhance follicular development. Very soon after the first IVF attempt Controlled Ovarian Stimulation (COS) was introduced in order to obtain a multi-follicular response of the woman ovary. The use of agents blocking the signalling by the Gonadotropin Releasing Hormone (GnRH) pulse generator towards the pituitary, such as GnRH agonists or GnRH antagonists, allows the controlled stimulation of follicular growth using a tailored dose of exogenous gonadotropins. In fact, follicles in the antral stage are sensitive to the Follicle Stimulating Hormone (FSH) signalling which promote follicular growth and the transition to the preovulatory stage. Follicular growth is usually monitored by circulating Estradiol (E2) measurement and transvaginal ultrasound (US) examination every second day from stimulation day 7. Once at least two follicles reach 18 mm of size in presence of appropriate E2 levels, ovulation process is triggered by the administration of human Chorionic Gonadotropin (hCG) which mimic the Luteinizing Hormone (LH) peak inducing final oocyte maturation. The presence of the first polar body (PBI) is normally considered to be a marker of oocyte nuclear maturity. The entire procedure was developed to reduce the risk of having no available oocyte and, at the same time, to obtain several mature oocytes, and thus valuable embryos, and replacing in the uterus the best one to improve the pregnancy chance (ESHRE Guidelines, 2019). US-guided oocyte retrieval (Oocyte Pick Up, OPU) is performed by follicular puncture and follicular fluid aspiration 36 hours after ovulation induction under local anaesthesia (paracervical block). Ovarian response is usually classified as poor, normal, and high. High ovarian response is a response to conventional ovarian stimulation characterized by the presence of

more follicles and/or oocytes than intended (Griesinger, et al., 2016). Generally, more than 18 follicles ≥11 mm in size on day of oocyte maturation trigger and/or 18 oocytes collected characterize a high response defined by an increased risk in Ovarian Hyperstimulation Syndrome (OHSS). Low ovarian response is a diminished response characterized by the presence of a low number of follicles and/or oocytes. Generally, ≤ 3 follicles on day of oocyte maturation trigger and/or ≤ 3 oocytes obtained characterize a low response (Ferraretti, et al., 2011). Sperm concentration, motility, and morphology is currently assessed according to the World Health Organization guidelines (WHO laboratory manual 5th ed., 2010). The retrieved cumulus-oocyte complexes (COCs) are placed in appropriate culture dish containing pre-equilibrated medium in incubators at 37°C and controlled atmosphere of 5% O2, 6% CO2, balanced with N2 (Figure 3).

Figure 3. Cumulus–oocyte complex consisting of a mature (methaphase II, MII) oocyte surrounded by expanded cumulus cells. The corona cells immediately adjacent to the oocyte become less compact after ovulation and radiating away from the oocyte membrane (Zona Pellucida, ZP). The first polar body (PBI) is located at the 1 o'clock position.

A limited number of oocytes is inseminated using partners' sperm to generate embryos in vitro, and supernumerary oocytes, if any, may be cryopreserved for further use. Fertilization

of all available oocytes is achieved using either conventional In Vitro Fertilization (IVF) or Intra-citosplasmic Sperm Injection (ICSI) within 4 hours after oocytes collection and according to sperm quality, number of previous fertility treatments and duration of infertility. IVF is performed in case of good quality of the sperm and consists in the incubation of sperm and egg at a ratio of 50,000:1 in the same culture dish until fertilization take place. Differently, the ICSI technique is used to overcome severe male infertility when sperm number and/or motility is low and implies that a sperm cell is injected directly into the egg cell using a specific microscope (Palermo et al., 1992). In addition, ICSI may be used in case of microsurgically collected sperm from the epididymis and the testis of azoospermic patients (Palermo et al.1995), to overcome previous fertilization failure by IVF (Palermo et al.1999), or for fertilization of oocytes that were previously cryopreserved as cryostress can lead to zona hardening (Porcu et al. 1997). The occurrence of normal fertilization, visible by the presence of the two pronuclei and the extrusion of the second polar body, is assessed 16-18 hours after insemination. The fertilized eggs (the zygotes) are kept in culture for 2-6 days in controlled atmosphere. During the in vitro culture 1 or 2 best embryos are replaced in uterus (embryo transfer, ET) after morphological evaluation either at the cleavage stage (4 cells on day 2 of development, 8 cells on day 3) or at the blastocyst stage (day 5) using a soft catheter under transvaginal US guidance. Supernumerary good quality embryos, if any, may be cryopreserved for further use. A pregnancy test checking for the presence of the hCG in the urine can be performed either 11 or 15 days after transfer of blastocyst or cleavage stage embryos, respectively.

The primary endpoint of an IVF treatment is a single live birth of a healthy baby. According to the data provided by the last registry of ESHRE, after IVF the clinical pregnancy rates (PRs) per oocyte retrieval and per transfer was at 28.5 and 34.6%, respectively. After ICSI, the corresponding PR achieved per OPU and per transfer was of 26.2 and 33.2%,

respectively. After frozen embryo replacement (FER) the observed PR per thawing was of 29.2%. The increased tendency of the transfer of fewer embryos in both IVF and ICSI resulted in a proportion of singleton, twin and triplet DR of 83.1, 16.5 and 0.4%, respectively (European IVF-monitoring Consortium (EIM), 2020).

Conversely, the principal side effect of IVF treatments is represented by Ovarian Hyperstimulation Syndrome (OHSS), an iatrogenic complication caused by the excessive release of the Vascular Endothelial Factor (VEGF) under the extensive stimulation of the ovary by gonadotropins. VEGF is a key substance that induces vascular permeability, leading to a shift of fluids from the intravascular system to the abdominal cavity. Symptoms may include abdominal pain, nausea, diarrhoea, and slight weight gain at a mild, moderate, and severe stage (Timmons et al., 2019). The proportion of IVF cycles complicated by OHSS decreased to 5.3 per 1,000 from 2006 to 2015 and the risk is highest for cycles with more than 30 oocytes retrieved. In singleton pregnancies, OHSS was associated with increased risk of low birth weight and preterm delivery. In twin pregnancies, OHSS was associated with an increased risk of second-trimester loss, low birth weight, and preterm delivery (Schirmer et al., 2020).

1.1.3 Morphological assessment of the human embryo

The success of an in vitro fertilization (IVF) treatment is the result of the balanced cooperation between the clinician and the embryologist. While the controlled ovarian stimulation aims at the retrieval of a good number of metaphase II (MII) oocytes, the correct gametes and embryos handling during the culture is of equal significance to determine the pregnancy chance. Embryo implantation is a process affected by several parameters, such as the embryo selection, the uterine receptivity and the transfer technique itself. Over the past 40 years, clinical and laboratory protocols became more efficient and IVF success rates have improved remarkably (Niederberger et al., 2018). As a consequence, IVF treatments are oriented on the optimization of the transfer policy regarding the time and the number of embryos to be transferred in utero with the aim of limiting the risk of multiple pregnancies while improving the pregnancy chance. The ability to identify the embryo with the highest potential to implant is of primary importance but is still a challenge in human assisted reproduction. In fact, despite active research in the field, embryologists select embryo for transfer depending on developmental rate and in-house morphological scoring systems based on light microscopy assessment (reviewed by Montag et al., 2011). As a consequence, morphological assessment represented the gold standard for selecting the best embryo for transfer (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). Embryos have been assessed morphologically ever since the start of IVF, which resulted in the generation of various morphological scoring systems based both on clinical tradition and evidence-based medicine (Edwards et al., 1981; Hill et al., 1989; Desai et al., 2000; Holte et al., 2007). However, implantation rate varies between 30% and 50% according to the embryo developmental stage, so that recently new approaches have been developed with the aim to improve embryo selection but, to date, none have been proven superior to standard morphological evaluation (Figure 4) (Sigalos et al., 2016).

Embryo selection technique	Study	Outcome
Extended culture to blastocyst stage	Blake et al. Cochrane Database Syst Rev, **2007** Oct 17; (4): p. CD002118 [9]	Higher PR and LBR in BC stage group VS cleavage stage in good prognosis patients with sufficient number of 8-cell embryos on day 3
	Glujovsky et al. Cochrane Database Syst Rev, **2012**. Jul 11; (7): p. CD002118 [10]	Higher LBR in BC group VS cleavage stage group in fresh ET. Higher cumulative (fresh + frozen embryos) PR from cleavage stage embryos VS BC stage
	Glujovsky et al. Cochrane Database Syst Rev. **2016** Jun 30;6:CD002118 [11]	Low quality evidence for LBR and moderate evidence that CPR are higher in BC group VS cleavage stage group in fresh ET. Unclear whether the day of the ET affects cumulative LBR and CPR
Embryo morphokinetics	Polanski et al. Ultrasound Obstet Gynecol. **2014** Oct;44(4):394–401 [32]	TLI has no effect on CPR or on OPR when blastocysts were transferred
	Kaser et al. Hum Reprod Update. **2014** Sep-Oct;20(5):617–31 [33]	There are insufficient data to support the use of TLI routinely in embryology lab
	Armstrong et al. Cochrane Database Syst Rev. **2015** Feb 27;(2):CD011320 [31]	There is not high level evidence that TLI can improve CPR and LBR when compared with conventional incubation
Metabolomics	Vergouw et al. Hum Reprod. **2014** Mar;29(3):455–61 [42]	NIR spectroscopy (in its current form) cannot be used to analyze spent culture media to increase LBR
Cumulus cells gene expression	N/A	N/A
Preimplantation genetic diagnosis for aneuploidies (PGD-A)	Lee et al. Hum Reprod. **2015** Feb;30(2):473–83 [79]	PGD-A screened embryos have higher IR than morphologically screened embryos in good prognosis patients. When single embryo transfer with PGD-A screening is performed in young good prognosis patients, higher CPR is expected
	Dahdouh et al. Reprod Biomed Online, **2015**. 30(3): p. 281–9 [81]	PGD-A after BC biopsy is correlated with higher IR and OPR when equal number of embryos are transferred in PGD-A and control group of good prognosis patients. PGD-A improves embryo selection in eSET cycles and decreases multiple pregnancy rates
	Chen et al. PLoS One, **2015**. 10(10): p. e0140779 [80]	PGD-A after cleavage or BC biopsy is correlated with higher IR, CPR, OPR and LBR when compared with traditional morphological methods

IR implantation rate, *CPR* clinical pregnancy rate, *OPR* ongoing pregnancy rate, *LBR* live birth rate, *BC* blastocyst, *ET* embryo transfer, *TLI* time lapse imaging, *PGD-A* preimplantation genetic diagnosis for aneuploidies, *eSET* elective single embryo transfer

Figure 4. Novel technologies proposed to improve embryo selection.

Since human embryonic development follows a specifically timed, coordinated sequence of events, developmental rate (assessed by the number of cells present at particular points in time) and morphological characteristics (defined at specified intervals after the day of insemination) have provided the two main morphological measures of embryonic development. However, morphological assessment still remains highly affected by inter- and intra- observer variation, so that no consistency and reproducibility is now guaranteed among the different IVF laboratories. As a consequence, the European Society of Human Reproduction and Embryology (ESHRE) have recently provided consensus points to define the minimum criteria for oocyte and embryo morphology assessment (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). It was

agreed that standardized timing of observations is critical to the ability to compare results between different laboratories, and that this should be related to the time of insemination (Figure 5 and 6).

Type of observation	Timing (hours post-insemination)	Expected stage of development
Fertilization check	17 ± 1	Pronuclear stage
Syngamy check	23 ± 1	Expect 50% to be in syngamy (up to 20% may be at the 2-cell stage)
Early cleavage check	26 ± 1 h post-ICSI 28 ± 1 h post-IVF	2-cell stage
Day-2 embryo assessment	44 ± 1	4-cell stage
Day-3 embryo assessment	68 ± 1	8-cell stage
Day-4 embryo assessment	92 ± 2	Morula
Day-5 embryo assessment	116 ± 2	Blastocyst

Figure 5. Timing of observation of fertilized oocytes and embryos and expected stage of development at each time point.

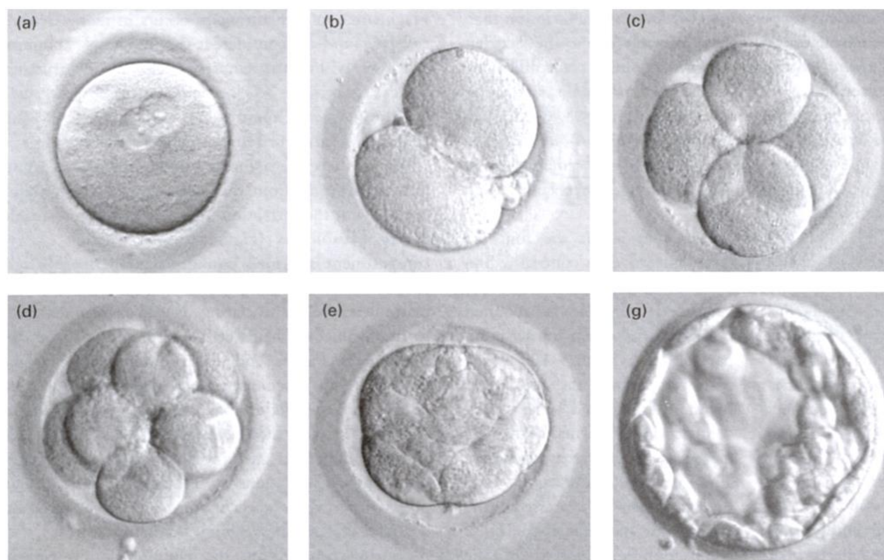

Figure 6. Preimplantation embryo development with pronuclear fusion (a), cleavage stage (b-d), compaction of the morula (e) and formation of the blastocyst (g).

Cleavage-stage embryo scoring system

For cleavage stage embryos on day 2 or 3 of development, the following features should be evaluated to determine embryo morphology: cell number, degree of fragmentation, equality of cell size and shape of blastomeres and presence of multinucleation. The consensus established that an optimal Day-2 embryo should have 4 equally sized mononucleated blastomeres in a three-dimensional tetrahedral arrangement, with <10% fragmentation. An optimal Day-3 embryo should have 8 equally sized mononucleated blastomeres, with <10% fragmentation. As a consequence, embryos that have a slower kinetic may have a reduced implantation potential, as well as embryos that have cleaved faster than the expected rate are likely to be abnormal and have a reduced implantation potential. Fragmentation is defined as the presence of cellular debris within the embryo volume and the result of cell cycles events. The relative degrees of fragmentation were defined as mild (<10%), moderate (10 – 25%) and severe (>25%). As fragments can move within the embryo volume, the definition of the impact of fragments could not be included in the standard morphological evaluation. Multinucleation is defined as the presence of more than one nucleus in a blastomere, including micronuclei, and the observation of multinucleation in one cell is sufficient for the embryo to be considered to be multinucleated. The consensus was that multinucleation is associated with a decreased implantation potential as these 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. Regarding cell size, for embryos at the 2-, 4- and 8-cell stages, blastomeres should be even sized. For all other cell stages, it is possible to expect a size difference in the cells, as the cleavage phase has not been completed. The grading scheme for cell size should be binary, noting whether all cell sizes are stage appropriate. Additional morphological features, such as cytoplasmic appearance, membrane thickness and the

presence of vacuoles, can also be noted as part of the morphological assessment on Day-2 and Day-3. The consensus scoring system for cleavage-stage embryos is showed in Figure 7.

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

Figure 7. Consensus scoring system for cleavage-stage embryos (in addition to cell number).

Blastocyst-stage scoring system

Morphological assessment of the blastocyst comprises the stage (early, expanding, expanded, hatching or hatched) as well as the quality of the inner cell mass and trophectoderm. A correlation between blastocyst morphology and implantation chance has been shown since these parameters started to be evaluated (Balaban et al., 2000, 2006). The most used scoring system for blastocyst assessment is the one proposed by Gardner and Schoolcraft (1999a, b; Gardner et al., 2000). This system, relies on three evaluation criteria, the expansion of the blastocoel, the number and organization of cells in both the inner cell mass (ICM) and the trophectoderm (TE). A top-quality blastocyst on day 5 would be a fully expanded blastocoel with an ICM that is prominent, easily discernible and consisting of many cells, with the cells compacted and tightly adhered together, and with a TE that comprises many cells forming a cohesive epithelium. It was agreed that both the ICM and a functional TE have a high prognostic value for implantation and foetal development. The consensus for the blastocyst assessment scoring system was that the evaluation should be a combination of stage and quality (Figure 8). The 'hatching' process is defined as the

emergence of part of the TE through the zona pellucida just before embryo implantation. According to the consensus scoring system ICM and TE should be graded with a 1–3 scale, with Grade 1 equivalent to Gardner A. In case of a blastocyst is collapsed at the time of assessment, it should not be graded immediately and re-evaluated 1–2 h later, as repeated cycles of collapse and re-expansion of blastocysts is currently observed.

	Grade	Rating	Description
Stage of development	1		Early
	2		Blastocyst
	3		Expanded
	4		Hatched/hatching
ICM	1	Good	Prominent, easily discernible, with many cells that are compacted and tightly adhered together
	2	Fair	Easily discernible, with many cells that are loosely grouped together
	3	Poor	Difficult to discern, with few cells
TE	1	Good	Many cells forming a cohesive epithelium
	2	Fair	Few cells forming a loose epithelium
	3	Poor	Very few cells

Figure 8. Consensus scoring system for blastocysts.

Definition of a non-viable embryo

It was the consensus opinion that a non-viable embryo is an embryo in which development has been arrested for at least 24h, or in which all the cells have degenerated or lysed. In addition, this definition has been recently implemented by the Italian Society of Embryology, Reproduction and Research (SIERR) together to the Italian Society of Human Genetics (SIGU), according which a non-viable embryo is not able to survive up to the postnatal life. This may occur also in case of a nuclear status different from the diploidy (number of pronuclei ≠2) at the zygote stage or in presence of a status of complex aneuploidy (two or more autosomes), partial or complete monosomy, partial or complete trisomy of specific chromosomes (1, 2, 3, 4, 5, 7, 10, 11, 12, 14, 19), aploidy or polyploidy, lethal monogenic disease at the blastocyst stage (www.sierr.it).

1.1.4 Cleavage stage vs. blastocyst transfer

Following the first live birth in 1978 (Edwards, 1981), laboratory protocols have been optimized with the aim to improve IVF outcome. This included both advancements in culture medium development (Nastri et al., 2016; Sfontouris et al., 2016) and transfer of embryos at different developmental stages. In the clinical practice embryo transfer (ET) may be performed either at the cleavage-stage (on day 2 or day 3 of development) or at the blastocyst stage (on day 5 or 6). In the clinical tradition, two embryos have been usually transferred at the cleavage stage aiming at increasing the likelihood of pregnancy, based on the evidence that each embryo has around 30% chance of implantation (Holte et al., 2007). However, the double embryo transfer (DET) may lead to an increased probability of multiple pregnancy, in particular di-zygotic twins (Andersen et al., 2008). Multiple pregnancy represents the biggest risk to both women and baby health in an IVF treatment, as twin pregnancy is frequently associated with a significantly higher risk of complications, such as prematurity, perinatal morbidity and increased perinatal mortality, as well as increased health service costs (Sebire et al., 2001). As a consequence, the elective single embryo transfer (e-SET) policy was suggested in order to prevent the risk of multiple pregnancy and the neonatal and obstetric-associated complications. The meta-analysis published by Gelbaya et al., (2010) reported that the e-SET of embryos at the cleavage stage reduced the likelihood of live birth by 38% and multiple birth by 94%, suggesting that three e-SET attempts (fresh plus frozen) resulted in a cumulative live birth rate similar to that of DET. In the last decades, elective single blastocyst transfer has been introduced and progressively became a widespread clinical strategy to reach a fairly good pregnancy rate with very low risk of twin pregnancy (Glujovsky et al., 2016). This practice is usually selected in women with good prognosis (age <35 years) with an adequate number of good-quality embryo (between 4 and 5) at the cleavage stage, where extended embryo culture to the blastocyst stage allows improved

embryo selection for transfer, thus potentially boosting the pregnancy rate per embryo transferred (Martins et al., 2017). In fact, the transfer at the blastocyst stage is considered to be a more physiologically appropriate time as it is closer to the time of natural implantation and improve synchrony between the endometrium and embryo development. Furthermore, by extending the duration of culture for an additional 2–3 days (from day 3 to day 5), embryo self-selection will occur after activation of the embryonic genome on day 3. This represents the natural selection process of the embryos capable of reaching the blastocyst stage and, theoretically, those embryos with the highest implantation potential (Racowsky et al., 2003; Machtinger et al., 2013). Because of these benefits, there is currently a trend to move from cleavage stage to blastocyst stage embryo transfer (Maheshwari et al., 2016). However, this approach decreases the total number of usable embryos (defined as those transferred plus frozen) as the in-vitro environment is inferior to that in vivo, which may lead to some embryos failing to blastulate in culture that would have implanted successfully if transferred at the cleavage stage (Kovalevsky et al., 2013; Racowsky et al., 2000). In addition, is still a matter of debate whether the extended in vitro culture beyond embryonic genomic activation of human embryos may influence the epigenetic profile (Mani et al., 2018). More importantly, there are concerns regarding the safety of blastocyst transfer, resulting associated foetal safety, such as increased preterm birth (PTB) and birth defects, as well as increased costs (Maheshwari et al., 2013; Dar et el., 2014).

Glujovsky et al., (2016) published a systematic review of 27 RCT comparing cleavage stage and blastocyst transfer. It was reported a significantly higher live-birth rate per couple when using fresh embryos transferred at the blastocyst stage compared with the earlier cleavage stage (odds ratio [OR] = 1.48; 95% confidence interval [CI], 1.20–1.82; day 2 to 3: 30.3%; day 5 to 6: 39.1%) (Figure 9) and a significantly higher clinical pregnancy rates after fresh

blastocyst transfer (OR = 1.30; 95% CI, 1.14–1.47; day 2 to 3: 37.2%; day 5 to 6: 43.2%). There was no evidence of a difference in miscarriage rates.

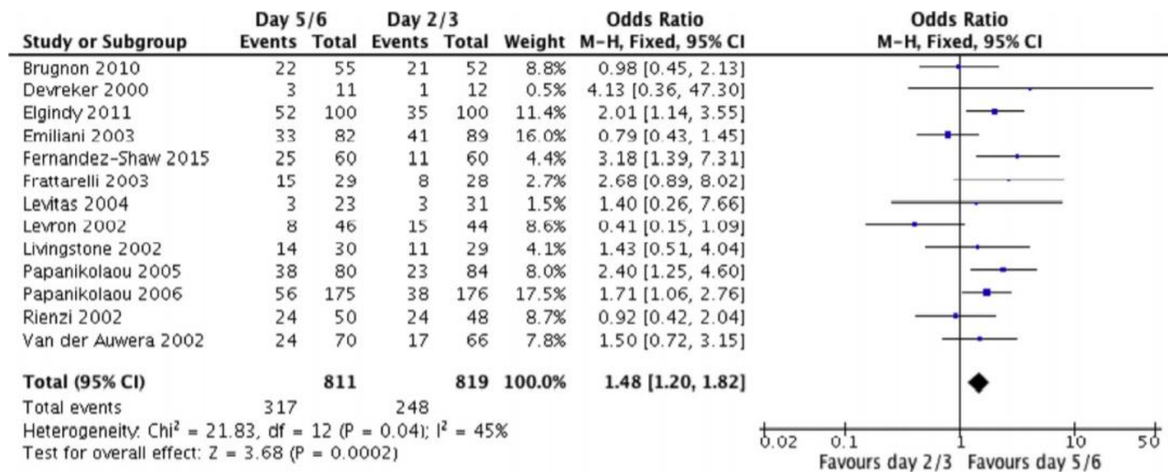

Figure 9. Live-birth rate in fresh cleavage-stage transfers and fresh blastocyst transfers.

There was no difference in the cumulative pregnancy rates between cleavage-stage and blastocyst transfer (OR = 0.89; 95% CI, 0.64–1.22; day 2 to 3: 48.9%; day 5 to 6: 52.0%) (Figure 10).

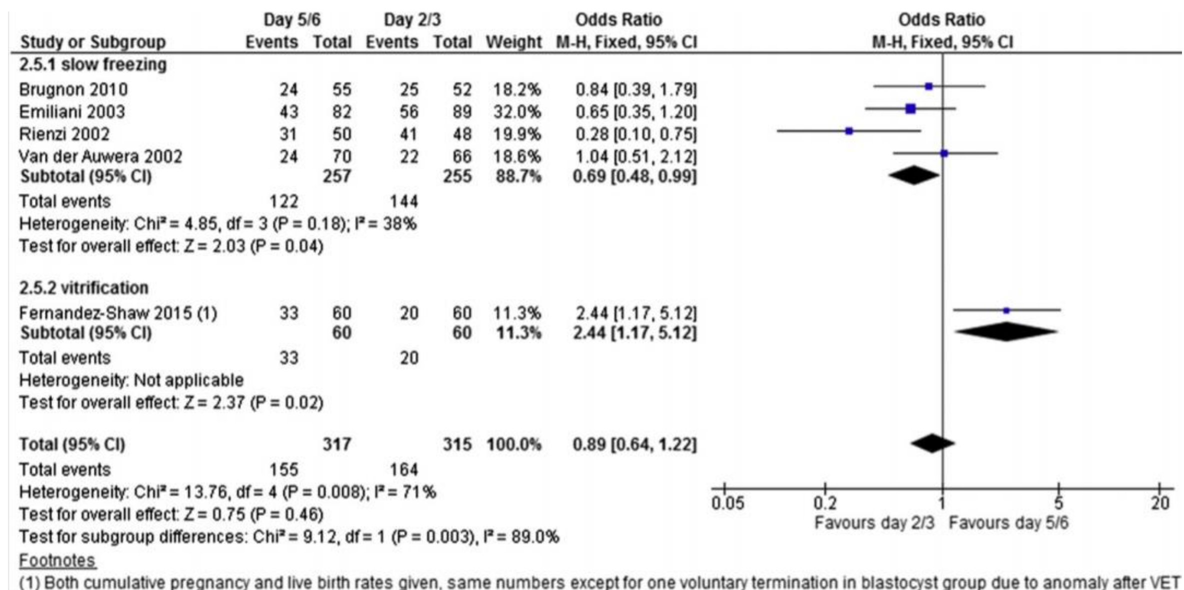

Figure 10. Cumulative pregnancy rate for fresh and frozen cleavage-stage transfers and blastocyst transfers grouped by freezing techniques.

A reduced number of cryopreserved embryos (OR = 0.48; 95% CI, 0.40–0.57) and an increased proportion of failure to transfer any embryos (OR = 2.50; 95% CI, 1.76–3.55; day 2 to 3: 3.6%; day 5 to 6: 8.5%) were also observed in case of blastocyst-stage transfer. This is due to the fact that not all embryos successfully develop to blastocyst stage, thus transferring cleavage-stage embryos and freezing the remainder for later transfer obviously provides couples more opportunities to transfer and more ETs, but this does not necessarily result in improved cumulative pregnancy rates. No economic analyses of the two different strategies have been reported, and it is currently unclear which strategy is more cost-effective.

Comparing the perinatal outcomes of singleton pregnancies for cleavage- and blastocyst-stage ETs, blastocyst transfer was associated with an increase in preterm birth before week 37 and with fewer small for gestational age babies (relative risk [RR] = 0.82; 95% CI, 0.77–0.88) with an absolute risk reduction of 1%–2%. The frequency of monozygotic twins was 1.6% in the blastocyst group and 0.4% in the cleavage-stage group (OR = 3.04; 95% CI, 1.54–6.01), although the difference was not statistically significant (Figure 11).

Currently, there is low-quality evidence that fresh blastocyst transfer is associated with improved live-birth rates and very low-quality evidence showing no significant differences in the cumulative pregnancy rate between cleavage-stage and blastocyst transfer, especially as extended culture is associated with an increased likelihood that no embryos will be available for either transfer or freezing and further assisted reproduction cycles will be required. To date, more than 35 years have elapsed and the optimal developmental stage for embryo transfer is still a subject of debate.

Outcome (ref.)	Evidence favors	Magnitude	Quality of the evidence[a]	Conclusions
Live-birth rate (5)	Fresh blastocyst	20%–82% higher with blastocyst transfer[b]	Low	Live-birth rate appears to increase with fresh blastocyst transfer. One in every 11 women who receive blastocyst have an additional live birth.
Cumulative live-birth rate (5)	Blastocyst	9%–478% higher with blastocyst transfer	Low	Only one study reported cumulative live birth data (authors provided additional data).
Cumulative pregnancy rate (5)	No difference	64% lower to 22% higher	Very low	The four studies that used slow freezing reported improved outcomes with cleavage stage, but the one study that used vitrification reported improved outcomes with blastocyst transfer.
Miscarriage rate (5)	Neither	–	Low	There was no evidence of a difference in miscarriage (18 RCTs).
No embryos available to transfer (5)	Cleavage	76%–355% higher with blastocyst transfer	Moderate	Nearly 9% of cycles with blastocyst transfer had no embryos to transfer, compared with 3.6% with cleavage-stage transfer.
Embryo freezing rate (5)	Cleavage	33%–60% lower with blastocyst transfer	Low	63% of cleavage-stage cycles had embryos to freeze, compared with 41% of blastocyst cycles.
Preterm birth (<37 wk) (18)	Cleavage	22%–31% higher with blastocyst transfer[c]	Low	Preterm birth may increase with blastocyst transfer. However, evidence comes from observational studies.
Very preterm birth (<32 wk) (18)	Controversial	–	–	No conclusions can be drawn. Two different systematic reviews of observational studies show controversial results.
Small for gestational age (19, 20)	Blastocyst	12%–23% lower with blastocyst transfer	Low	Small for gestational age may decrease with blastocyst transfer. However, evidence comes from observational studies.
Monochorionic twins (21)	Controversial	–	–	Although it may increase with blastocyst transfers, this was not seen in newer studies that used sequential culture media.

[a] According to GRADE classification (17).
[b] If analyzing only studies from 2005 onwards, it is 32%–135% higher.
[c] In the original publication, it is reported as a higher frequency in blastocyst stage instead of lower frequency in cleavage stage.

Glujovsky. Cleavage-stage versus blastocyst transfer. Fertil Steril 2016.

Figure 11. Summary of the Cochrane review findings.

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1.2 Time-Lapse technology (TLT)

Time-lapse (TL) is a cinematographic, motion-picture technique by which a naturally slow process can be seen at a greatly increased rate. The technique involves recording a single film frame at intervals of lapsed time but when the full sequence of images is subsequently played back as a movie, the event is speeded up in proportion to the interval between frames. For example, an image of a scene may be captured at 1 frame per second, but then played back at 30 frames per second; the result is an apparent 30 times speed increase. As a consequence, processes that would normally appear slow to the human eye (e.g. the motion of the sun and stars in the sky or the growth of a plant) become very accelerated.

Studying biological systems, time-lapse microscopy represents a method to extend live cell imaging from a single observation in time to the observation of cellular dynamics over long periods of time. Microscope image sequences are recorded and then viewed at a greater speed to give an accelerated view of the microscopic process. The first reported time-lapse microscope was assembled in the late 1890s at the Marey Institute (Marseilles), founded by the pioneer of chorophotography, Étienne-Jules Marey. However, Jean Comandon, a trained microbiologist specializing in syphilis research, was the first who made significant scientific contributions in 1910 studying the movements of the syphilis bacteria. In fact, using a cinema camera coupled with the microscope, he demonstrated visually that the movement of the disease-causing bacteria is uniquely different from the non-disease-causing form thus providing evidence on how to distinguish the two forms (Bayly, 1910; Figure 12). Comandon's extensive pioneering work inspired others to adopt microcinematography. During World War II, Carl Zeiss AG released the first phase-contrast microscope able to observe cellular details without using lethal stains for the first time. By performing some of the first time-lapse experiments with chicken fibroblasts, Michael Abercrombie described

the basis of our current understanding of cell migration in 1953. The broad introduction of digital cameras coupled to microscopy made time-lapse technology dramatically more accessible to investigate dynamic processes in different research fields of biology and medicine (Burton, 1962).

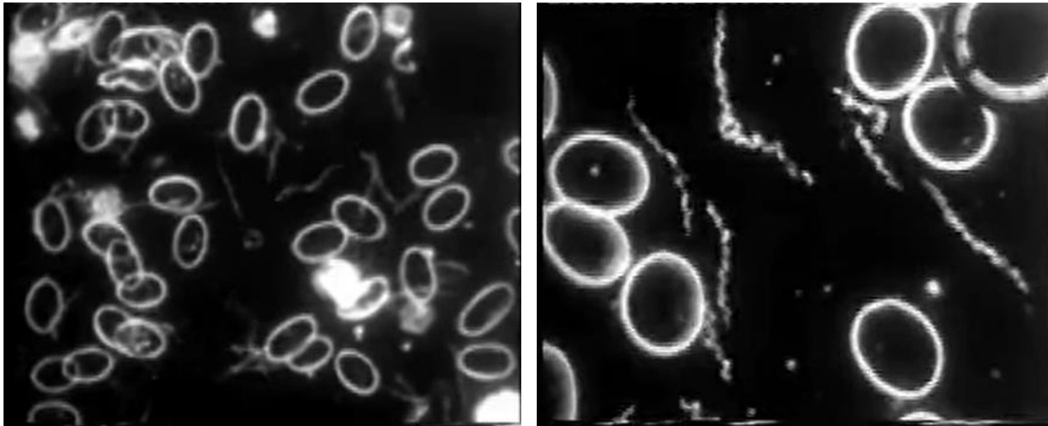

Figure 12. These two video frames show living syphilis spirochaetes moving among red blood cells of frog. The back-and-forth movement observed characterizes disease-causing spirochaete. The original video is believed to be the oldest preserved time-lapse microscopy video.

1.2.1 Impact of Time-Lapse technology on human reproduction

The developmental competence and implantation potential of the human embryo is daily performed by the assessment of morphological features at static time points, providing a 'snap-shot' of embryo development. However, this approach has a limited ability to accurately predict embryo quality and pregnancy chance (Guerif et al., 2007), although the attempt to standardize the morphological assessment across different laboratories through the consensus on the timings and characteristics of morphological features of human embryos published by ESHRE and Alpha Scientists in Reproductive Medicine (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). Unfortunately, the limitations of static morphology evaluation were not overcome. In addition, morphological evaluation is also affected by inter- and intra- observer variation, so that limited consistency and reproducibility is guaranteed among different IVF laboratories (Storr et al., 2017). Furthermore, it usually requires physical removal of the embryos from the incubator, exposing them to fluctuations in temperature, pH and oxygen levels. As a consequence, the improvement of non-invasive embryo selection strategies and the maintenance of an optimal incubation environment represent two crucial factors for the successful outcome of IVF treatments. ù

Impact of TLT on culture conditions

During in vitro culture embryos are constantly exposed to physical and chemical stressors that they would not experience in vivo. Sources of stress include pH and temperature variations, exposure to atmospheric oxygen (20% O_2) tension and the accumulation of toxic components in the culture media originating from both embryo metabolism and volatile organic compounds (Wale and Gardner, 2016). Since oocytes and embryos are highly sensitive to microenvironment variations, that may dramatically impact the developmental

potential also post transfer in uterus, improvements in culture conditions still remain of primary importance to optimize IVF treatment.

Culture media to support embryo development to the blastocyst stage have been based on two distinct approaches, the sequential and the single step medium approach (Machtinger and Racowsky, 2012; Quinn, 2012). The first approach was designed to meet the metabolic shift occurring in the mammalian embryo after the embryonic genome activation on day 3 (Morin et al., 2017). In this case, embryos are cultured from days 1–3 in a first growth medium and then, at the cleavage stage on day 3, are moved to a second medium that promote blastocyst formation. The second approach aims at supporting the embryo development with a single nutrient composition for the entire preimplantation period from days 1 to days 5/6, allowing developing embryos to choose the nutrients they require, while at the same time minimizing embryo handling for medium refresh on day 3. Data obtained in conventional incubators revealed inconclusive evidence on whether one culture system is superior to the other (Sepulveda et al., 2009; Sfontouris et al., 2016; Werner et al., 2016). The application of time-lapse technology to embryo assessment naturally involves the use of a single step approach to ensure minimal embryo handling and an uninterrupted growth during the entire in vitro culture. Data comparing embryo morphokinetic behaviour between sequential and single-step media resulted to be conflicting, as some authors observed a significant faster kinetic in the early cleaving embryos in the single step group (Ciray et al., 2012; Kazdar et al., 2017), not confirmed by other studies (Basile et al., 2013). Overall, no clear consensus has been reached on the benefits of uninterrupted embryo culture neither on morphokinetics nor on implantation and live birth often because of confounding variables (Sfontouris et al., 2016; Costa-Borges et al., 2016; Alhelou et al., 2018; Swain et al., 2019; Figure 13).

Benefits	Risks
Reduced dish removal from incubator: more stable culture conditions (gas, temperature)	Media degradation (ammonia production, substrate depletion and other component degradation)
Reduced cell handling: reduced risk for cell damage or loss	Missing possible important embryo morphological indicators or signs of potential culture issues (only in non-time lapse incubators)
Accumulation of beneficial autocrine and paracrine factors	Volatile organic compound accumulation (in oil and or media)
Compatible with new time-lapse incubators: new technology and additional selection end-points	Media evaporation: osmolality increase, pH increase and increase in other solute concentrations (in dry incubators)
Improved workflow: less staff time and possible cost savings	Mineral oil degradation (peroxidation)

Figure 13. Potential benefits and risks of an uninterrupted embryo culture.

Thus, data to date have been unable to demonstrate conclusive superiority of neither single-step nor sequential media in terms of clinical outcomes when used in conjunction with TL incubators.

Multiple studies have described that the in vivo oxygen tension in the female reproductive tract of mammalian species is between 2 and 8% (Fischer and Bavister, 1993). For decades, the majority of clinical embryology has been performed at atmospheric O_2 (20%), but it has been observed that this may increase the production of reactive oxygen species (ROS) (Yang et al., 1998) thus altering gene expression (Rinaudo et al., 2006), DNA methylation profile (Li et al., 2016) and embryo metabolism (Wale and Gardner, 2012). Multiple meta-analyses now exist demonstrating improved pregnancy rates and live birth rates with 5% oxygen (Bontekoe et al., 2012; Nasri et al., 2016). For these reasons lower O_2 tension is also recommended to TL incubators. Data provided by Kirkegaard et al., (2013) suggested that atmospheric O_2 reduces developmental rates and delays completion of the third cell cycle at the cleavage stage. Since evidence is still limited and inconclusive to date is not recommended to draw conclusion on the impact of oxygen level on human embryo morphokinetics.

Human embryos can be cultured in vitro either in groups or individually, while the embryos of many other mammals specifically require culture in groups. For instance, it has been observed that mouse embryos are more sensitive to the accumulation of atmospheric O_2 when cultured individually. In fact, grouping more embryos in the same culture dish may lower local O_2 concentrations and, as a consequence, reduce ROS formation in the culture media (Wale and Gardner, 2010; 2012). In addition, paracrine factors secreted by embryonic metabolic processes may play a critical role in supporting growth during group culture (Ebner et al., 2010). Kelley and Gardner (2016) analysed the morphokinetic behaviour of mouse embryos using TLT to evaluate the combined impact of embryo density and O_2 levels in the culture medium, observing that embryos showed a significantly delayed kinetic if cultured in 20% oxygen individual culture compared to 5% O_2 group culture, persisting to the blastocyst stage. Furthermore, the blastocyst hatching rate and the number of cells of the inner cell mass (ICM) of the generated blastocysts were reduced in 20% oxygen individual culture, indicating a combined detrimental effect of individual culture and 20% oxygen on embryo development (Kelley and Gardner, 2017). However, the design of the culture dishes currently used in the IVF laboratories does not allow the ideal group culture. In fact, two types of dishes can be used even in different time-lapse systems, one having multiple microwells under one drop of media, and the other with a single well which require separate drops of media. There is evidence that the multiple microwell type better supports embryo development compared with single culture in individual drops (Chung et al., 2015).

Impact of TLT on embryo assessment

Time-lapse technology was introduced in clinical embryology in 1997 applied to the investigation of polar body extrusion and pronuclear formation (Payne et al., 1997), but it was not until 2010 that it was used as a selection and prediction tool. At first, Wong et al.

(2010) described that human blastocyst formation may be predicted by the observation of cell division timings on day 2 of development before embryonic genome activation. One year later, Meseguer et al. (2011) showed that embryo implantation resulted associated with specific cell division timing parameters, introducing the term 'morphokinetics' in the IVF laboratory. Time-lapse systems typically comprise a stand-alone incubator with one or more integrated inverted microscopes coupled to a digital camera. Digital images of embryo development are recorded at regular intervals of 5–15 min and at different focal planes and subsequently processed into videos allowing embryologists to assess embryo development in a dynamic, real-time manner without removing them from the incubator (Montag et al., 2014; Figure 14).

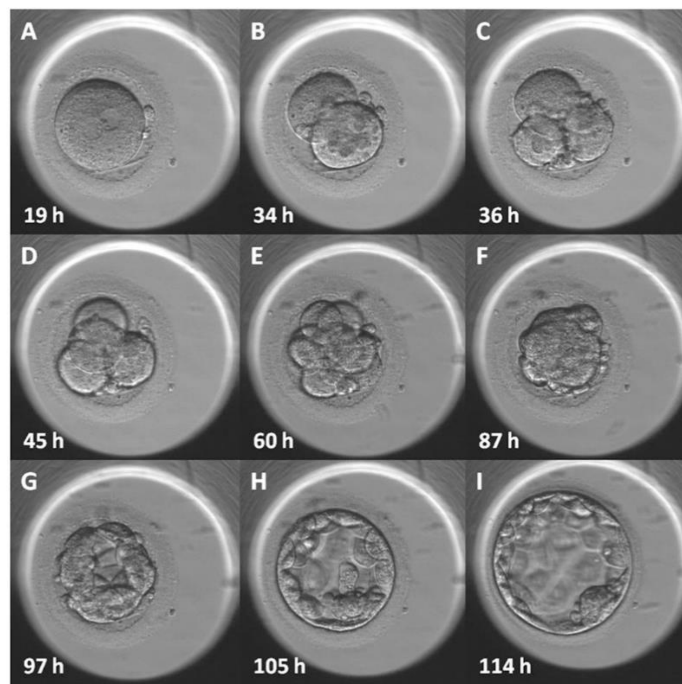

Figure 14. Frames form a time-lapse observation of the human embryo development.

For this reason, time-lapse technology may also represent an excellent tool for teaching embryology and standardizing assessment. In fact, the dynamic observation of unusual/abnormal cleavage events can be used as learning material of embryo developmental pattern. Also, the time factor is no longer an issue in the embryo evaluation as the complete

analysis of the videos generated can be performed a posteriori. As a consequence, the gradual introduction of TLT in the clinical practice may imply two main benefits: i) the dramatic increase in the number of observations during embryo culture to perform a dynamic assessment of embryo development; ii) the maintenance of undisturbed culture environment, minimizing embryo handling and the need to expose embryos to suboptimal conditions outside of the incubator (Meseguer et al., 2012). Another important advantage provided by time-lapse observation is represented by the evidence of a more limited intra- and inter-observer variability among different embryologists performing the video analysis, providing a more objective, consistent and reliable embryo evaluation and an overall improved inter-laboratory agreement on clinical decisions (Sundvall et al., 2013; Martinez-Granados et al., 2017; Martinez et al, 2018). Taken together, this evidence highlights the reliability of time-lapse assessment, but they cannot still be considered conclusive. In fact, automated annotation of embryo morphokinetic still requires human supervision to correct inaccuracies that may affect the embryo selection process. Drawback of this crucial aspect is the requirement of specific training programs for the laboratory staff on using this type of device and an accurate quality control system.

1.2.2 Morphokinetic annotation of the human embryo development

The identification of the embryo with the best implantation potential still remains an unresolved issue in human reproduction. This is the result of the limited predictive capacity of the static embryo morphology evaluation, which is routinely performed on a restricted number of time points and of the evidence that a large proportion of the embryos in culture do not exactly follow the expected developmental timing (Gardner, 2015). In addition, the static assessment of embryo quality implies that no information is provided regarding the development between these time points, and significant events may be missed. For these reasons new recording systems capturing images with time-lapse devices have been developed to allow a noninvasively 24 hours monitor of embryo development (Kirkegaard et al., 2012). The continuous monitoring is performed by using cameras incorporated in the incubation chamber to dramatically increase the quantity and quality of information regarding embryo developmental kinetic without disturbing culture conditions. This would include the complete evaluation of abnormal cell divisions such as direct cleavage and reverse cleavage which would be undetectable with static embryo assessment (Rubio et al., 2012; Liu et al., 2014). Some atypical cleavage anomalies have been described exclusively by using TL monitoring and correlated with embryo quality and implantation potential (ESHRE Working group on Time-lapse technology, 2020) (Figure 15).

Name of feature	Explanation	Observed exclusively or better by TLT	References
Abnormal syngamy	Erratic PN movement in the cytoplasm	Exclusively	(Athayde Wirka et al., 2014, Coticchio et al., 2018)
Asynchronous appearance of two pronuclei	Disappearance of one and appearance of another pronucleus	Exclusively	(Coticchio et al., 2018)
Differently sized pronuclei	Difference in pronuclear areas immediately before pronuclear membrane fading	Exclusively	(Otsuki et al., 2017)
Pronuclei reappearance	Pronuclei fading and reappearance	Exclusively	(Coticchio et al., 2018)
Aberrant behaviour of female pronucleus	Extrusion of the third PB instead of female pronucleus formation	Exclusively	(Mio et al., 2014)
Fragmentation of pronuclei	Formation of micronuclei	Better	(Mio et al., 2014)
Fusion of pronuclei	A pronucleus formed by the fusion of two preexisting pronuclei	Exclusively	(Mio et al., 2014)
Unipolar cleavage furrow	Appearance of cleavage furrow on one site of the zygote	Exclusively	(Hojnik et al., 2016, Wong et al., 2010)
Tripolar cleavage furrow	Appearance of three cleavage furrows on the zygote	Exclusively	(Wong et al., 2010)
Pseudofurrows	Zygote presenting oolemma ruffling before cytokinesis	Exclusively	(Athayde Wirka et al., 2014, Wong et al., 2010)
Absent cleavage	Arrest in zygote stage despite normal fertilisation	Better	(Barrie et al., 2017)
Direct cleavage	Cleavage of zygote to three cells (trichotomous mitosis) or one blastomere to three cells in the first (t3-t2 = 0) or second cell division cycle (two cells to five or six cells), but this should be distinguished from rapid cleavage (t3-t2 < 5 h)	Exclusively	(Athayde Wirka et al., 2014, Barrie et al., 2017, Fan et al., 2016, Lagalla et al., 2017, Meseguer et al., 2011, Rubio et al., 2012, Zhan et al., 2016)
Reverse cleavage	Fusion of two cells into one blastomere	Exclusively	(Barrie et al., 2017, Desai et al., 2014, Goodman et al., 2016, Liu et al., 2014b)
Blastomere movement	Prolonged blastomere movement induced by delay in pronuclear fading and first cell division	Exclusively	(Ezoe et al., 2019)
Multinucleation	Blastomere with > 1 nucleus	Better	(Balakier et al., 2016, Desai et al., 2014, Ergin et al., 2014, Goodman et al., 2016, Hashimoto et al., 2016)
Internalization of cellular fragments	Fragments reabsorbed into one mother blastomere	Exclusively	(Hardarson et al., 2002, Mio et al., 2014)
Irregular chaotic division	Disordered cleavage behaviour with uneven cleavages and fragmentation	Better	(Athayde Wirka et al., 2014, Barrie et al., 2017)
Early compaction	Formation of tight junctions between blastomeres in day 3 or even day 2 embryos	Better	(Iwata et al., 2014, Le Cruguel et al., 2013)
Cell exclusion	Exclusion of one or more blastomeres from the formation of compact morula or blastocyst	Better	(Coticchio et al., 2019, Lagalla et al., 2017)
Blastocyst collapse	Complete or almost complete disappearance of blastocoel and consequent blastocyst shrinkage	Better	(Bodri et al., 2016b, Kovacic et al., 2018, Marcos et al., 2015)

Figure 15. Atypical human embryo cleavage features observed with time-lapse technology versus classic embryo morphology assessment once per day.

Guidelines were proposed on the nomenclature and annotation of the events observed during embryo development followed with a TL system (Ciray et al., 2014). The variables and the description of the events and intervals are summarized in Figure 16 (ESHRE Working group on Time-lapse technology, 2020).

	Terminology	Description of the event
	tPB2	The second polar body is completely detached from the oolemma
	tPNa	Appearance of individual pronuclei; tPN1a, tPN2a, tPN3a, . . .
	tPNf*	Time frame of pronuclei fading; tPN1f; tPN2f...
	tZ	Time of PN scoring (last time frame before tPNf)
	tn*	First time frame at which an embryo reaches *n* number of blastomeres (e.g. t2, t3, t4)
	tTM	Trichotomous mitosis at different stages
	tSC	First evidence of compaction
	tM	Time of completion of compaction process (in case some blastomeres are excluded, it might be difficult to assess the real time frame)
	tSB	Initiation of blastulation (first frame in which the blastocoel is visible)
	tB	Full blastocyst (last frame before zona starts to thin)
	tE or tEB	Initiation of expansion; first frame of zona thinning (also called TEyB 'y' corresponds to morphology of inner cell mass; 'z' corresponds to morphology of trophectoderm cells)
	tHN	Herniation; end of expansion phase and initiation of hatching process (also called tHNyz)
	tHD or tHB*	Fully hatched blastocyst (also called tHDyz)
Dynamic events and time intervals	Psyn	Syngamy, time from PN fading to the first cytokinesis
	Not mentioned	Time between nuclear envelope breakdown and subsequent division to two cells
	s2	Time between division to three cells and subsequent division to four cells
	s3	Time between division to five cells and subsequent division to eight cells
	ECC1	Duration of the first cell cycle (t2-tPB2)
	cc2	Blastomere cell cycle: Duration of the second cell cycle (*a* = t3-t2, *b* = t4-t2)
	cc3	Blastomere cell cycle: Duration of the third cell cycle (*a* = t5-t4, *b* = t6-t4, *c* = t7-t4, *d* = t8-t4)
	ECC2	Embryo cell cycle: t4-t2
	ECC3	Embryo cell cycle: t8-t4
	Blastocyst contraction	A decrease in blastocoel volume
Cryopreserved/ warmed blastocyst	tRE	Time of the start of re-expansion (first frame in which the blastocoel reforms or increases in size)
	tCRE	Time of completion of re-expansion (first frame the blastocyst occupies the whole perivitelline space)

*Figure 16. Nomenclature of morphokinetics parameters. *Parameters with the highest concordance between operators.*

Depending on the type of the TL system adopted, some events may not be seen. Time zero (t0) may change from one study to another (mid-time for ICSI, standard IVF insemination, tPB2 or tPNf). These inconsistencies have to be taken into account when comparing data from different studies. Thus, studies using time-lapse technology may represent a novel strategy to introduce new dynamic markers of embryonic competence. Markers of embryo quality at early stages of development are of particular interests to clinics where extended embryo culture to the blastocyst stage is not feasible for different reasons. Morphokinetic

events occurring during embryo culture can be annotated and analysed using integrated computer programs with the aim to facilitate embryologists in the ranking of embryos based on their developmental ability. When choosing embryos for transfer/cryopreservation, laboratories with TLT will be able to implement their selection/deselection method or ranking strategy more confidently and incisively. Since the introduction of TLT, several studies aimed at evaluating the clinical and biological significance of the parameters listed in Figure 15. A non-exhaustive summary of these studies is depicted in Figure 17 (ESHRE Working group on Time-lapse technology, 2020).

Markers	**Prediction/outcome**	**Reference**
Time interval cytoplasmic halo appearance ➔ disappearance	Embryo quality on day 3	(Coticchio *et al.*, 2018)
Time interval halo appearance ➔ PN fading	Embryo quality on day 3	(Coticchio *et al.*, 2018)
Time interval PN fading ➔ first cleavage (t2)	Embryo quality on day 3	(Coticchio *et al.*, 2018)
Time interval male PN appearance ➔ male PN fading	Embryo quality on day 3	(Coticchio *et al.*, 2018)
PNs movement and fading	Blastocyst formation	(Athayde Wirka *et al.*, 2014)
Appearance of nuclei after first cleavage	Pregnancy success	(Lemmen *et al.*, 2008)
Duration of the first cytokinesis	Blastocyst formation	(Wong *et al.*, 2010)
Time interval between the end of the first mitosis and the initiation of the second	Blastocyst formation	(Wong *et al.*, 2010)
Time interval between the second and third mitoses	Blastocyst formation	(Wong *et al.*, 2010)
tPNf	Live birth	(Azzarello *et al.*, 2012)
	Implantation	(Aguilar *et al.*, 2014, Chamayou *et al.*, 2013, Kirkegaard *et al.*, 2013c, Wu *et al.*, 2016)
tPB2	Implantation	(Aguilar *et al.*, 2014)
Length of s-phase	Implantation	(Aguilar *et al.*, 2014)
t2	Implantation	(Meseguer *et al.*, 2011, Mizobe *et al.*, 2016a, Wu *et al.*, 2016)
	Blastocyst formation	(Mizobe *et al.*, 2018)
	Top-quality blastocyst formation	(Mizobe *et al.*, 2016a)
	Embryo quality on day 3	(Coticchio *et al.*, 2018)
t3	Implantation	(Meseguer *et al.*, 2011)
t4	Implantation	(Carrasco *et al.*, 2017, Freour *et al.*, 2013, Meseguer *et al.*, 2011, Mizobe *et al.*, 2016a, Wu *et al.*, 2016)
	Top-quality blastocyst formation	(Mizobe *et al.*, 2016a)
t5	Implantation	(Meseguer *et al.*, 2011)
t6	Top-quality blastocyst formation	(Storr *et al.*, 2015)

Parameter	Significance	Reference
t7	Implantation	(Carrasco et al., 2017)
	Top-quality blastocyst formation	(Storr et al., 2015)
t8	Implantation	(Dal Canto et al., 2012)
	Top-quality blastocyst formation	(Storr et al., 2015)
tn	Implantation	(Chamayou et al., 2013, Kirkegaard et al., 2013c)
Mean duration of two-cell stage	Implantation	(Meseguer et al., 2011) (Rubio et al., 2012)
	Expanded blastocyst formation	(Dal Canto et al., 2012)
	Blastocyst development	(Conaghan et al., 2013, Cruz et al., 2012, Wong et al., 2010)
Mean duration of three-cell stage	Implantation	(Meseguer et al., 2011)
	Blastocyst development	(Conaghan et al., 2013, Cruz et al., 2012, Wong et al., 2010)
	Expanded blastocyst formation	(Dal Canto et al., 2012)
tM	Top-quality blastocyst formation	(Storr et al., 2015)
	Blastocyst formation and implantation	(Chamayou et al., 2013, Kirkegaard et al., 2013c, Motato et al., 2016)
	No difference in implantation	(Chamayou et al., 2013, Kirkegaard et al., 2013c)
tSC	Implantation	(Chamayou et al., 2013, Kirkegaard et al., 2013c)
tSB	Top-quality blastocyst formation	(Fishel et al., 2018, Storr et al., 2015)
	Implantation	(Goodman et al., 2016, Mizobe et al., 2017)
tB	Top-quality blastocyst formation	(Storr et al., 2015)
	Implantation	(Chamayou et al., 2013, Kirkegaard et al., 2013c)
tEB	Blastocyst formation and implantation	(Motato et al., 2016)
	Implantation	(Chamayou et al., 2013, Kirkegaard et al., 2013c)
s3	Blastocyst formation	(Cetinkaya et al., 2015)
	Top-quality blastocyst formation	(Storr et al., 2015)
	Blastocyst formation and implantation	(Motato et al., 2016)
	Implantation	(Carrasco et al., 2017, Chamayou et al., 2013)
cc3	Implantation	(Chamayou et al., 2013)
Blastocyst contraction	Implantation rate	(Marcos et al., 2015, Vinals Gonzalez et al., 2018)
tRE, tCRE	Pregnancy	(Ebner et al., 2017)
Post thawing blastocyst re-expansion speed (tCRE-tRE)	Pregnancy and pregnancy loss Live birth	(Ebner et al., 2017) (Kovacic et al., 2018)

Figure 17. Morphokinetic parameters with biological/clinical significance.

TLT and embryo ploidy

Embryo ploidy status is one of the most critical factors affecting the embryo's implantation potential. The preimplantation genetic testing of embryo aneuploidies, also known as PGT-A, allows the identification of embryo chromosomal status. However, this technique suffers from some limitations, due to regulatory issues in some countries (Harper et al., 2014), it can be considered invasive, it requires specific technical skills, it can take up to several days before obtaining the result according to the genetic technique used, it still remains expensive (Gardner et al., 2015; Sermon et al., 2016) and, more importantly, there remains some debate regarding its cost-effectiveness and/or clinical relevance (Penzias et al., 2018; Rosenwaks et al., 2018; Somigliana et al., 2019). Aiming at providing cheaper, faster and less invasive approaches for the evaluation of embryo ploidy status than PGT-A, embryo morphokinetics was postulated to be associated to the chromosomal status (Campbell et al., 2013). Based on the data reported by a recently published systematic review of the literature comprising 13 studies, none of the morphokinetic parameters considered may provide robust evidence for the recommendation of the clinical use of TLT for embryo ploidy assessment (Reignier et al., 2018). This evidence was further supported by other contemporary studies where TLT was suggested for the selection of the embryo with the highest implantation potential in conjunction to PGT-A (Zaninovic et al., 2017; Rocafort et al., 2018).

1.2.3 Morphokinetic algorithms for human embryo selection

As previously mentioned, many morphokinetic parameters were correlated with human embryo development and implantation (Figure 16) and may be considered biomarkers of embryo viability to perform a more accurate embryo selection for transfer or cryopreservation. Recently, these parameters have been used to develop embryo-selection algorithms seeking at combining morphological and morphokinetic parameters predictive of blastocyst formation, implantation and live birth (Petersen et al., 2016; Carrasco et al., 2017). The first selection algorithm was developed by Meseguer et al. (2011), later improved in a larger dataset by Basile et al. (2015). They elaborated a hierarchical classification of embryos, from A to D, based on: (i) morphological assessment; (ii) new morphological criteria (direct cleavage from one to three cells, uneven blastomere size in second cell cycle and multinucleation in third cell cycle); (iii) timing of cell division to three cells (t3); (iv) duration of second cell cycle (cc2), corresponding to the time from division to a two blastomere until division to a three blastomere embryo; (v) timing of cell division to five cells (Figure 18).

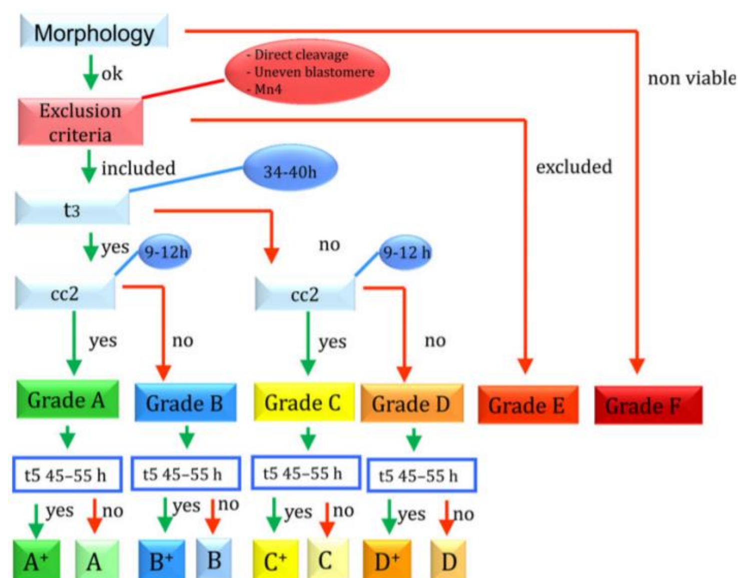

Figure 18. Hierarchical classification combining morphology and kinetic criteria subdividing embryos according to implantation potential.

More recently, the efficacy of six published algorithms for predicting embryo viability was compared by Barrie et al. (2017) in terms of implantation rate (IR), suggesting the need for the development of in-house models that are specific to the patient, treatment, and environment, as many confounder factors should be taken into account, such as age, dose of FSH administered, years of infertility, fertilization method and culture conditions (Kirkegaard et al., 2016). As a consequence, these data suggest that currently available algorithms may not be clinically applicable to all laboratories and lose their diagnostic value when externally applied (Figure 19).

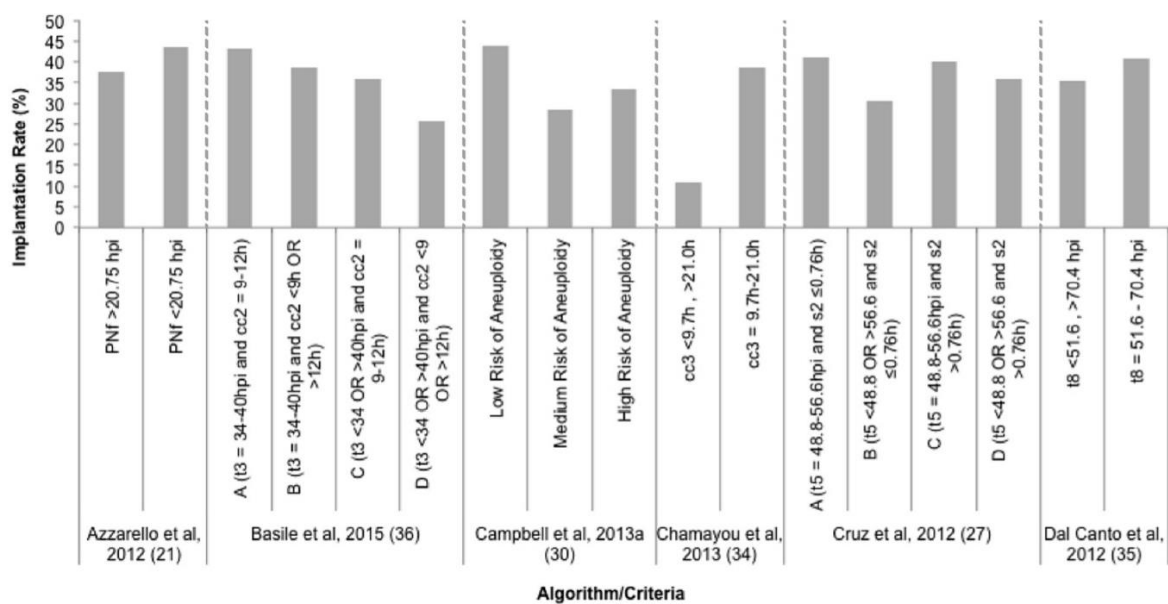

Figure 19. Implantation rates (IRs) of the embryo classification categories in each of the analysed embryo selection algorithms.

1.2.4 Clinical benefit of Time-Lapse technology on IVF treatments

Although there are several studies that support the hypothesis that TLT may represent a useful strategy to identify the best embryo for transfer, more research is required to improve the quality of the current evidence (Kaser et al., 2014; Castelló et al., 2016). Before the introduction in the IVF clinical routine, robust studies are needed to confirm the safety and efficacy of this adjunct treatment or 'add-on' (Harper et al., 2017). In fact, TLT should be tested not only for blastocyst formation or implantation rate, but also for live birth, miscarriage and twinning rates and obstetric/perinatal problems (Armstrong et al., 2015). In addition, ethical aspects and cost-effectiveness evaluation should not be excluded. To date, clear evidence of increased success rates with the use of TLT remains to be provided. A putative benefit of TLT was suggested by Pribenszky et al. (2017) reporting significantly higher ongoing pregnancy and live birth rates and a significantly lower early pregnancy loss. Conversely, these data were not confirmed by other meta-analyses (Polanski et al., 2014; Chen et al., 2017; Magdi et al., 2019). The latest Cochrane review comprising 9 RCTs (2955 women) reported, again, insufficient evidence for differences in live birth rate (OR = 1.12, 95% CI 0.92–1.36), miscarriage rate (OR = 0.63, 95% CI 0.45–0.89) or clinical pregnancy rate (OR = 0.95, 95% CI 0.78–1.16) for TLT combined with embryo selection software versus conventional incubation and assessment (Armstrong et al., 2019). As the evidence is of low or very low quality, these findings should be interpreted with caution. The main reason for the controversy over TLT efficacy is the fact that it entails two distinct components, namely an undisturbed incubation environment and an embryo selection through imaging software. These two components have not been effectively distinguished in the majority of studies, possibly masking the weight of the effect of better culture conditions or improved embryo selection on IVF outcomes. Additional confounders that may explain the heterogeneity amongst studies include number of days of assessment,

different endpoints, the wide range of morphokinetic timings assessed, inter- and intra-operator variability in annotation. Importantly, comparing obstetric outcomes of singleton pregnancies obtained in patients with embryos cultured in a TL system with those with embryos grown in standard incubators, Insua et al. (2017) observed no differences respect to weeks at delivery, preterm births and very preterm births. Again, no statistical differences were found in neonatal outcomes such as birth weight, low birth weight and very low birth weight. No major malformations or perinatal mortality were found in either of the two groups (Figure 20).

This evidence suggested that TLT is an effective and safe alternative for embryo incubation, where continuous embryo monitoring in an undisturbed environment may offer more information on embryo development, compared with static observations, to enhance the identification of good-prognosis embryos for clinical use. In order to firmly establish a putative beneficial effect of TLT, more well-designed and sufficiently powered RCTs reporting on live births and perinatal outcomes are necessary.

Variable	TLS (n = 149)	SI (n = 122)	*P* value
Pregnancy outcome			
Maternal age (y)	35.6 (35.1–36.0)	37.4 (36.6–38.3)	
1st trimester bleeding	22 (14.8)	13 (10.7)	NS
Invasive procedures[a]	13 (8.7)	11 (9.0)	NS
Anemia (Hb <11 g/dL)	22 (14.8)	13 (10.7)	NS
Gestational cholestasis	5 (3.4)	5 (4.1)	NS
Diabetes	15 (10.1)	13 (10.7)	NS
2nd and 3rd trimester bleeding	9 (6.0)	5 (4.1)	NS
PROM <37 wk	13 (8.7)	8 (6.6)	NS
Pregnancy-induced hypertension	18 (12.1)	18 (14.8)	NS
Urinary tract infection	4 (2.7)	12 (9.8)	.013
Delivery outcome			
Weeks at delivery	38.8 (38.4–39.1)	39.5 (38.0–39.9)	NS
Preterm births (<37 wk)	16 (10.7)	15 (12.3)	NS
Very preterm births (<34 wk)	4 (2.9)	4 (3.3)	NS
Cesarean section	93 (36.9)	49 (60.5)	< .007
Puerperal problems	11 (4.4)	4 (4.9)	NS
Neonatal outcome			
Female neonates	114 (45.2)	51 (63.0)	< .006
Birth weight (g)	3,163 (3,035–3,292)	3,074 (2,913–3,236)	NS
Low birth weight (<2,500 g)	19 (12.8)	15 (12.3)	NS
Very low birth weight (<1,500 g)	3 (2.0)	3 (2.4)	NS
Neonatal height (cm)	50.3 (49.6–50.9)	49.7 (48.9–50.4)	NS
Apgar score at 1 min	9.1 (8.8–9.3)	8.9 (8.7–9.2)	NS
Apgar score at 5 min	9.7 (9.5–9.9)	9.8 (9.7–9.9)	NS
Apgar score at 10 min	9.9 (9.8–10)	9.9 (9.7–10.0)	NS
Malformations	1 (0.6)	0	NS
Major malformations	0	0	NS
Minor malformations	1 (0.6)	0	NS
Admission to NICU	8 (5.4)	3 (2.5)	NS
Days in the NICU	12.8 (1.1–24.6)	6.7 (0–17.8)	NS
Perinatal mortality	0	0	NS

Figure 20. Obstetric and perinatal outcome of IVF treatments using TLT.

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1.3. Artificial Intelligence (AI)

Artificial intelligence (AI) is defined as the use of complex algorithms to make machines to reason and perform cognitive functions, including problem solving and decision making. Alan Turing (mathematician, computer scientist, philosopher and theoretical biologist) was the first who described the possibility to use computers to emulate human neural processes like intelligent behaviour and critical thinking in 1950. In fact, Turing developed the so-called "Turing test" to determine whether computers were capable of human intelligence. Six years later, John McCarthy (computer and cognitive scientist) described for the first time the term artificial intelligence (AI) as "the science and engineering of making intelligent machines." Several decades of computer research developed many subfields in AI, such as machine learning (ML), deep learning (DL), natural language processing (NLP) and computer vision (CV) (Kaul et al., 2020).

1) *Machine Learning (ML)*: aims at the identification of specific patterns from the analysis of a data sets describing a particular situation. The machine can then "learn" from the submitted situation and apply that information to future similar scenarios for the development of predictive algorithms. Typical application of ML algorithms is clustering and feature identification, pattern recognition, classification and prediction.

2) *Deep Learning (DL)*: composed of algorithms combining the original features to create multi-layer artificial neural network (ANN) which enable machines to make decisions on its own identifying a higher level features that have far more predictive value than the original features.

3) *Natural Language Processing (NLP)*: process that enables computers to extract data from human language and make decisions based on that information.

4) *Computer Vision (CV)*: process by which a computer gains information and understanding from a series of images or videos.

Today, AI is used in daily life in different informatics areas including Internet search engines, social media news feeds, speech recognition in digital assistants like Siri and Alexa, face recognition for newest smartphones, tailored searches in Spotify, Netflix, Amazon, and YouTube, GPS navigation and self-driving vehicles. The introduction of AI in human pathology has dramatically increased over the past 5 decades, creating new opportunities for personalized medicine. The generation of predictive models may be applied to improve the accuracy in the diagnosis of diseases by the interpretation of medical images, optimize the therapeutic response to drugs classifying patient profiles, facilitate risk prevention and follow-up monitoring by predicting long term outcomes, improve training and education, increase clinical data collection (Angehrn et al., 2020; Rakha et al., 2020; Figure 21).

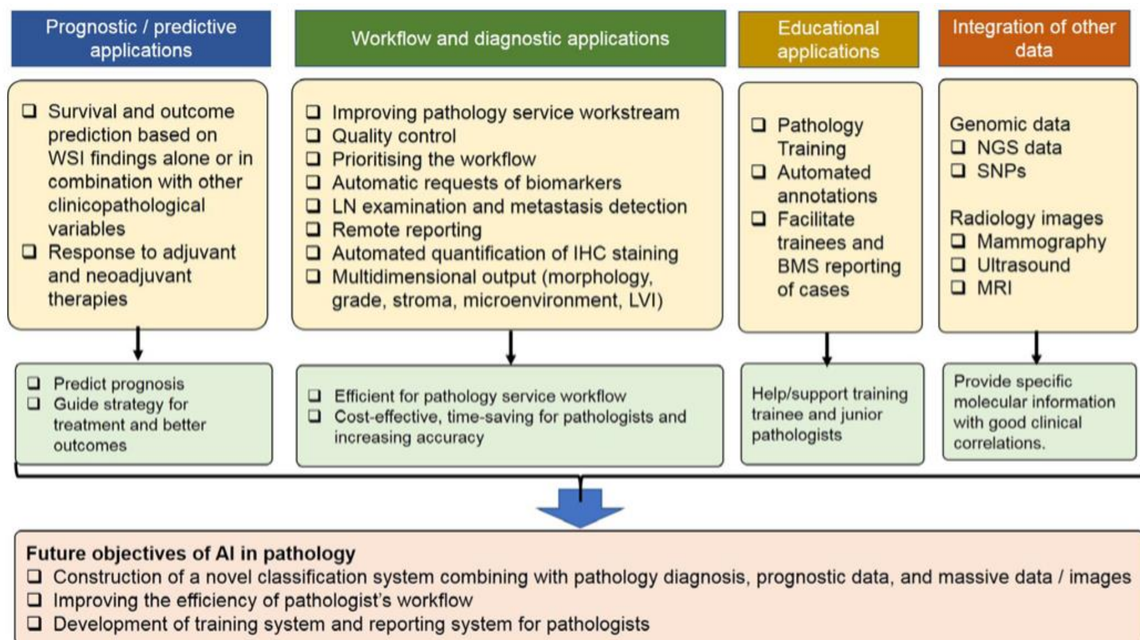

Figure 21. Diagram illustrating the main applications of AI in human patology.

Modern medicine has shifted from developing treatments after the fact, to preventing, personalising and delivering precision care by the integration of vast amounts of data including genomic biomarkers, clinical examinations and baseline characteristics of patients. AI is a novel tool to gather these data to achieve the vision of personalised medicine. As reviewed by Emin et al. (2019), artificial intelligence represents a promising tool also in obstetrics and gynaecology, as a means to improve knowledge and assist clinicians in decision-making. For example, ML models can be applied to cardiotocography (CTG), used to monitor the foetal heart and uterine contractions and functions to help to determine whether a caesarean section is necessary. AI algorithms may be use in gynaecological oncology to dissect multifactorial mechanisms involved in cancer progression in order to provide reliable and promising predictive markers of prognosis and response to treatments.

1.3.1 Role of Artificial Intelligence in reproductive medicine

In recent years, the attraction towards artificial intelligence (AI) and machine learning (ML) has quickly increased in human reproduction and embryology (Zaninovic et al., 2019, 2020). In fact, in just one year, published abstracts at the annual meeting of the American Society of Reproductive Medicine (ASRM) and European Society of Human Reproduction and Embryology (ESHRE) increased seven-fold (Curchoe et al., 2019). Advances in AI field are constantly promoted by the increasing amount of data available in reproductive medicine. The aim of AI or ML application is to help clinicians in the decision-making process, providing the most appropriate therapy, increasing the pregnancy chance and reducing both the time to pregnancy and the financial burden. In addition, AI is constantly implemented aiming at the identification of predictive markers to optimize the semen sample analysis and oocyte/embryo assessment, reduce errors and provide automatic classification algorithms able to improve the efficacy of ART treatments (Wang et al., 2019; Figure 22).

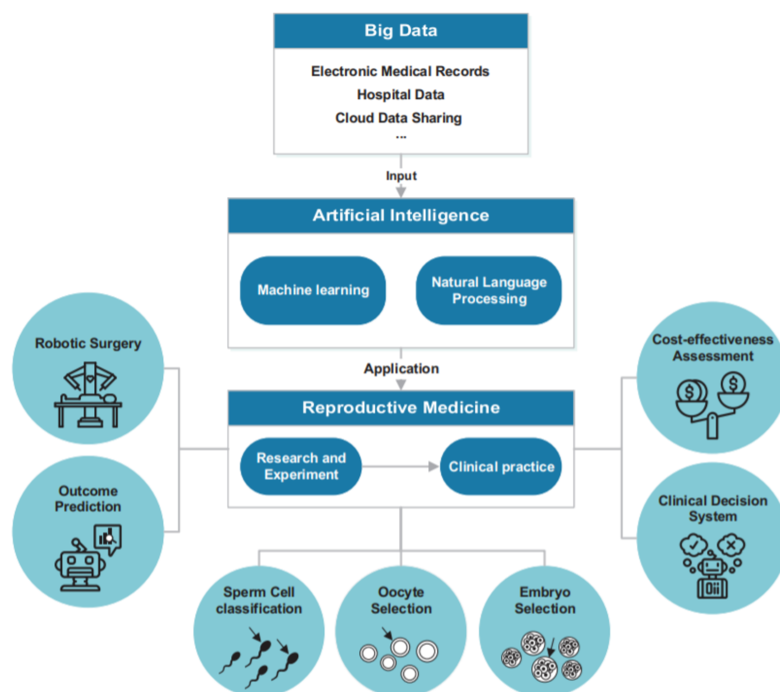

Figure 22. Review of the seven main applications of AI in reproductive medicine.

However, the key limiting factor of these studies is the quantity and quality of data, which significantly affect the performance, applicability and generalizability of the trained model (Fernandez et al., 2020). As previously described, several embryo selection strategies have been described in literature using a single or a restricted number of parameters obtaining only limited efficiency in terms of an increased pregnancy chance (Sigalos et al., 2016; Chavez-Badiola et al., 2020). In order to predict the probability of a successful pregnancy for each patient and to fully understand the cause of each failure, AI-based methods may represent an innovative strategy to solve this issue.

In recent years, AI was coupled to sperm sample analysis with the aim to minimize the variations between laboratories and standardize the evaluation of sperm motility and morphology. The computer-aided sperm analysis (CASA) system is an automated method based on image analysis developed to gain more objective and precise results in the clinical practice (Goodson et al., 2017). Also, neural networks have been developed using data of the lifestyle and environmental features obtained from questionnaires to make predictions for seminal quality (Girela et al., 2013). Sahoo & Kumar (2014) used five AI approaches to predict the fertility rate in humans and applied eight features selection strategies to find out appropriate attributes that can predict male fertility rate more accurately. Manna et al. (2013) used textures of microscopic images to develop an artificial neural network for the prediction of oocyte and embryo quality. Another example is represented by Yanez et al. (2016) who used a support-vector machine (SVM) classifier, learning models with associated algorithms for classification, based on zygote viscoelastic properties to predict blastocyst formation in humans and mice. As a result, embryo quality assessment performed by conventional morphology in conjunction with artificial intelligence algorithms represent an attractive possibility to improve embryo selection strategies in the IVF lab. Santos Filho et al. (2012) proposed a SVM classifier for semi-automatic grading of human blastocyst based on

microscope images of inner cell mass (ICM) and trophectoderm (TE) quality. By the analysis of the image textures, a morphological assessment of the blastocyst was provided. Saeedi et al. (2017) introduced the first automated approach to identify TE and ICM of human blastocysts via texture with an accuracy of 86.6% and 91.3%, respectively, thus providing a more objective assessment. More recently, embryo selection may be performed coupling conventional morphology to morphokinetic parameters using Time-Lapse technology for a dynamic monitoring of embryo development. Carrasco et al. (2017) retrospectively analysed the morphokinetic parameters of human embryos with known implantation data and developed a model for the prediction of the embryo implantation potential on day 3. AI may find application in reproductive medicine also in the generation of prediction model to help clinicians to tailor personalized treatments and improve the pregnancy chance in IVF (Simopoulou et al., 2018). Several models have been described, with accuracies ranging from 59% (Kaufmann et al. 1997) to 84.4% (Guvenir et al. 2015).

Among the different AI approaches, machine learning (ML) with decision tree (DT) and deep learning (DL) with Neural Networks (NN) are two of the most used in reproductive medicine (Wang et al., 2019; Goyal et al., 2020; Figure 23). DT is a classifier that forms a tree structure, which consists of various nodes, such as root nodes and leaf nodes or decision nodes. The decision nodes carry out several tests to predict the class label and each class is calculated to gain its probability. DT is generally the algorithm preferred by physicians as, compared to other algorithms such as neural network, it is easier to interpret and understand. Neural networks, inspired by neurons in the human brain, are the most commonly used algorithms for image analysis today. The ultimate objective of the overall neural networks is to learn the appropriate representations to arrive at an accurate prediction for new input data. The network employed consists of input layers (primary features), hidden layers (secondary features) and output layers (final outcome).

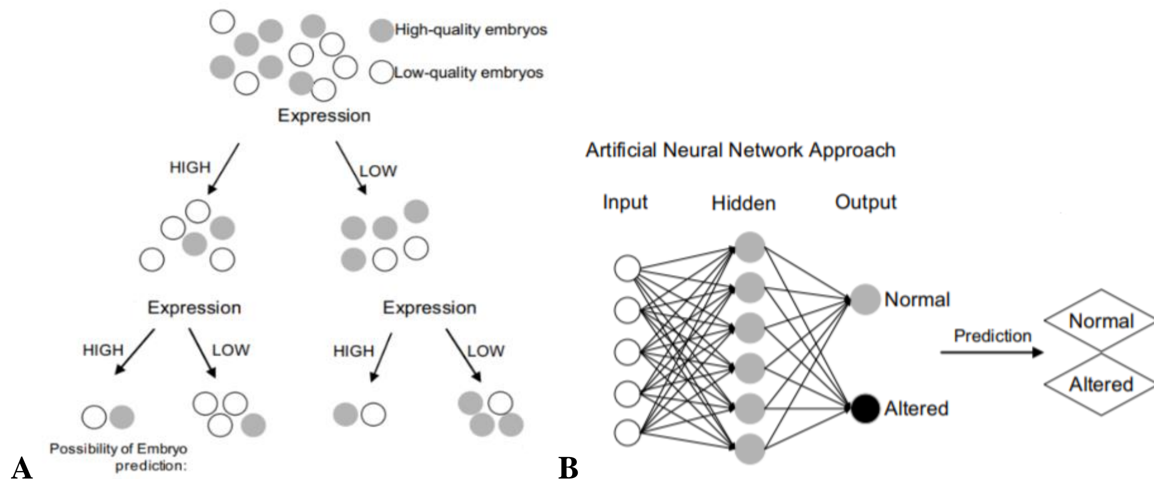

Figure 23. (A) Representative DT based on the combination of the expression of two different factors to make predictions for the selection of embryos. The grey colour represents high-quality embryos whereas the white colour represents low-quality embryos. (B) Example of an ANN to produce a decision-support system that may help to predict semen parameters to support the traditional diagnosis (modified from Wang et al., 2019).

Some ML models can be compared to a "black box", lacking a clear interpretation for scientists, clinicians and also patients. In addition, the performance of AI approaches is strongly related to the quantity and quality of the input data, as small datasets can lead to wrong decisions if selection bias are present during the sample collection. If correctly performed, machine learning can handle simple and repetitive tasks, helping clinicians in the decision-making process. However, they should not blindly follow the predictions of the ML models during the clinical practice but should always consider whether the model is constructed reasonably and it is compatible with the actual clinical routine (Wang et al., 2019). The future trend would be to create an interconnected network of patient data combining medical data, images, laboratory examinations, genetic information and health records with advanced AI methods to personalize medical diagnosis and treatments.

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2. Research project

2.1 Aim of the thesis and experimental plan

The major challenge in the field of embryology is to develop objective strategies able to identify which embryo, within a cohort, possesses the highest probability of resulting in a healthy live birth. Since the beginning of human IVF, embryo selection for transfer has relied on the assessment of static morphology, possessing a limited predictive power on embryo developmental potential. As a consequence, the identification of novel strategies to optimize early embryo selection and improve pregnancy chance has emerged as an urgent issue in modern reproductive medicine. The introduction of the Time-Lapse technology (TLT), allowing a dynamic and undisturbed analysis of embryo morphokinetics in vitro, has opened novel opportunity to identify new markers for embryo selection. In order to evaluate the beneficial effect of TLT in embryo grading, in our paper entitled *"Impact of the addition of Early Embryo Viability Assessment to morphological evaluation on the accuracy of embryo selection on day 3 or day 5: a retrospective analysis"* (Revelli et al., 2019) we assessed whether the addition of a time-lapse system to standard morphology may increase the accuracy of embryo selection in case of double embryo transfer (DET) on day 3 or single embryo transfer (SET) on day 5. Currently, the transfer of a single blastocyst in utero represents the standard of care in clinical routine of IVF in order to maximize the probability of a single ongoing pregnancy. In the attempt of finding a multivariable approach taking into account different issues regulating embryo developmental capacity to the blastocyst stage, we evaluated whether morphokinetic variables and the expression of specific genes in cumulus cells (CCs) could be independent predictors of embryo development. In our paper entitled *"Morphokinetic analysis of cleavage stage embryos and assessment of specific gene expression in cumulus cells independently predict human embryo development to expanded*

blastocyst: a preliminary study" (Canosa et al., 2020) we analysed the expression of specific genes involved in oocyte maturation and the morphokinetic features of the corresponding embryos at the cleavage stage comparing embryos that progressed to the expanded blastocyst stage on day 5, embryos that arrested their development and embryos that reached an intermediate state of development. In recent years, several predictive algorithms gathering different clinical and embryological variables have been proposed to further optimize the embryo selection process. However, classic statistics often failed in the generation of models taking into account links and associations among variables. The introduction of artificial intelligence (AI) in reproductive medicine, have opened a revolutionary scenario for the generation of complex algorithms handling an extremely large amount of clinical data. In particular, machine learning (ML), a subset of AI, allows computers to detect patterns automatically and uses them to make predictions. In our last work *"Development of a Machine Learning algorithm based on early morphokinetic for human blastocyst development prediction: a retrospective analysis of 575 cleavage-stage embryos" (Canosa et al., unpublished)* we developed a machine learning algorithm for human embryo classification based on early morphokinetic parameters obtained using a time-lapse system to improve the selection at the cleavage stage of embryos with the best chance to develop to the expanded blastocyst stage on day 5. To our opinion, the use of ML, and AI in general, may represent the future direction to personalize the infertility treatments and increase the pregnancy chance in IVF.

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Impact of the addition of Early Embryo Viability Assessment to morphological evaluation on the accuracy of embryo selection on day 3 or day 5: a retrospective analysis

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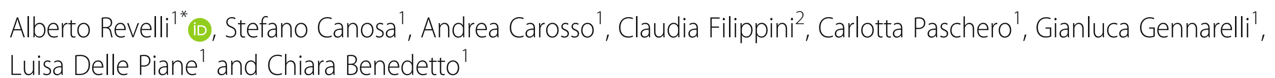

Abstract

In this study we aimed at retrospectively assessing in a homogeneous group of IVF patients whether the addition of Early Embryo Viability Assessment (Eeva™) to standard morphology increases the accuracy of embryo selection in case of double embryo transfer (DET) on day 3 or single embryo transfer (SET) on day 5. Eeva™ is an algorhythm aimed at indicating on day 3, according to morphokinetic parameters observed in the first three days of embryo growth, which embryos are more likely to develop into viable blastocysts and implant. A total number of 328 patients were included in the study; IVF or ICSI were performed and 428 embryos were transferred, either with DET on day 5, or (when at least four top scored embryos were available on day 3) with SET of day 5. Four groups were considered: (a) patients receiving day 3 DET with embryos selected by standard morphology (DET-3 M, $n = 106$, receiving 212 embryos), (b) patients receiving day 3 DET with embryos selected by morphology plus Eeva™ (DET-3 ME group, $n = 48$, receiving 96 embryos), (c) patients receiving day 5 SET with a blastocyst selected by standard morphology (SET-5 M group, $n = 126$, receiving 126 embryos), and (d) patients receiving day 5 SET with a blastocyst selected by morphology plus Eeva™ (SET-5 ME group, $n = 48$, receiving 48 embryos). Overall, a clinical pregnancy rate of 49.1%, implantation rate of 40%, and ongoing pregnancy rate of 43.6% were observed. The implantation rate was significantly higher in DET-3 ME group than in DET-3 M group (44.8% vs. 30.2%, $p < 0.02$), whereas it was comparable in groups DET-3 ME, SET-5 M and SET-5 ME. Differently, the ultrasound-verified clinical pregnancy rate and the ongoing pregnancy rate at 12 weeks did not significantly differ in all four groups. Overall, our findings suggest that Eeva™ algorhythm can improve embryo selection accuracy of standard morphology when ET on day 3 is scheduled, leading to a higher implantation rate, but its impact on ongoing pregnancy and live birth needs to be further clarified.

Keywords: Time lapse, Embryo score, IVF, Clinical pregnancy rate, Ongoing pregnancy rate

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Introduction

Selecting the best embryo(s) to be transferred in uteri in the same cycle or cryopreserved for a delayed transfer is a key issue in human IVF. In the last decades, embryo morphology was widely used for detecting embryo competence, and several morphological scores applying to cleavage stage (day 2 or 3) embryos were proposed, none of them, however, being adopted as a worldwide-accepted standard. The strategy of transferring a single embryo to avoid twin pregnancy was progressively adopted in the last years, and extended culture to the blastocyst stage, although rather complicated and time-consuming, became quite popular, raising the need for blastocyst morphological scores; also in this case, however, a thoroughly reliable method to select the best blastocyst was not yet found.

Time-lapse embryo culture technology (TLT) is a rather recent approach to embryo selection. It allows the continuous, dynamic assessment of embryo morphological changes without the need to remove embryos from the incubator, thus limiting potentially detrimental effects of changes in culture conditions [8]. To date, however, only some studies reported higher clinical and ongoing pregnancy rates using embryo selection by TLT vs. the classical morphological embryo selection [12], whereas other prospective randomized trials did not find any improvement in IVF outcome with TLT [5, 9, 10]. To date, a convincing, final demonstration that TLT can improve IVF results in terms of live births is still lacking.

Early Embryo Viability Assessment (Eeva™) is an algorhythm that applyes to a specific type of TLT system; it was designed to predict blastocyst formation on the basis of morphokinetic parameters observed in the first three days of embryo growth. Eeva™ was aimed at indicating on day 3 which embryos are more likely to develop into viable blastocysts, and gives the potential advantage to select the most competent embryos on day 3 without the need to extend culture till day 5, thus saving time and resources. Until now, however, only a few studies assessing the impact of Eeva™ on cleaving embryo selection were performed: a multicenter study concluded that the combination of standard morphology plus Eeva™ was more effective than standard morphology alone in identifying embryos able to reach the blastocyst stage [2]; other studies reported that Eeva™ application improved the possibility to select embryos and increased implantation and pregnancy rates [1, 16]. On the other side, however, some authors reported no significant differences in pregnancy rates when Eeva™ was used in association with standard morphology vs. morphology alone on day 3 [9] or vs. morphological selection alone on day 5 [7, 18].

In the present study we aimed at retrospectively assessing in a very homogeneously selected group of IVF patients whether the addition of Eeva™ to standard morphology increased the accuracy of embryo selection in case of double embryo transfer (DET) on day 3 or single embryo transfer (SET) on day 5.

Materials and methods

Patients

The study was designed as a retrospective analysis of IVF cycles performed at our IVF Unit between March 2016 and July 2018. In order to minimize the risk of interference by patient-related characteristics, we included in the study a very homogenous group of carefully selected patients chosen with the following, strict criteria: (a) age 28–40 years; (b) body mass index (BMI) 18–25); (c) ovarian reserve markers predicting a normal response to gonadotropins (serum day 3 FSH < 12 IU/l, AMH 1.2–5 ng/ml, antral follicle count (AFC) 8–15); (d) verified normal response to controlled ovarian stimulation (COS) performed by gonadotropin-releasing hormone (GnRH)-agonist ″long″ protocol plus recombinant FSH, with at least 6 retrieved oocytes; (e) easy transfer of two embryos on day 3 (DET) or (in case of at least 4 good-scoring embryos on day 3) of a single blastocyst on day 5 (SET).

The study was carried out in accordance to the Declaration of Helsinki and was authorized as an observational study by the local Ethical Committee. A signed, written consent was retrospectively obtained from all patients accepting their data being included in the analysis.

Controlled ovarian stimulation (COS) and oocyte retrieval

All included patients underwent COS using the gonadotropin-releasing hormone (GnRH)-agonist ″long″ protocol with recombinant FSH (Gonal-F®, Merck, Darmstadt, Germany) at individually tailored starting daily dose (125–250 IU/day, subcutaneously). Follicular growth was monitored by transvaginal US examination plus serial measurements of circulating estradiol (E2), performed every second day from stimulation day 7, and adjusting FSH dose accordingly. When at least two follicles reached 18 mm mean diameter, with appropriate E2 levels, a single subcutaneous injection of 10,000 IU hCG (Gonasi HP, IBSA, Pambio Noranco, Switzerland) was administered to trigger ovulation. US-guided oocyte retrieval (OPU) was performed 35–37 h later under local anesthesia (paracervical block). The aspirated follicular fluid was immediately observed under stereomicroscope to retrieve the corresponding oocyte, that was then washed in buffered medium and incubated in controlled atmosphere until fertilization procedure.

Preparation of semen samples and in vitro fertilization

Semen samples were examined to assess sperm concentration, motility, and morphology according to the

World Health Organization guidelines, and were then prepared by density gradient centrifugation in order to select normally motile, morphologically normal spermatozoa. Conventional IVF or ICSI injection was performed on all available oocytes within 4 h after OPU, and the occurrence of normal fertilization was assessed after 16–18 h of incubation in controlled atmosphere by evaluating the presence of two pronuclei (2PN) and the extrusion of the second polar body.

Embryo selection and transfer

Fertilized oocytes were placed in pools in a 4-wells dish (Thermo Scientific, Denmark), and were cultured in pre-equilibrated Cleavage medium (Cook) overlain with mineral oil, using the same tri-gas box incubators (Panasonic) containing an atmosphere of 5% O2 and 6–7% CO2, balanced with N2. A medium refresh step on day 3 was performed.

When only the classical morphological evaluation was applied, the Integrated Morphology Cleavage Score (IMCS) was used. IMCS is the only score constructed to be evidence-based, as it was obtained comparing implanted embryos vs. non-implanted embryos in a rather large number of IVF cycles ending in double embryo transfer (DET) [6]. Due to its peculiar characteristics, IMCS has been incorporated in a complex prediction model for IVF outcome, proven to predict live birth with a remarkably good accuracy [14]. After IMCS-based morphological selection, either a double embryo transfer (DET) on day 3 or a single blastocyst transfer (SET) on day 5 were performed. SET was chosen when at least 4 good scoring (> 8/10 at IMCS) embryos were available on day 3; in this case, the blastocyst morphological selection on day 5 was performed as previously described [15] and the transferred embryo was chosen taking into account both the IMCS score and the blastocyst morphological score.

When the Eeva™ system was added to standard morphology to assess embryo competence, embryos were cultured in microwells of the Eeva™ dish (12 wells/dish; one embryo/well), whose format allows following each embryo individually even if all embryos share a common 100 μL drop of medium (group culture). Embryos were cultured in pre-equilibrated Cleavage medium (Cook, Ireland) overlaid with mineral oil. Eeva™ microscopes were housed in tri-gas box incubators (Panasonic) containing an atmosphere of 5% O2 and 6–7% CO2, balanced with N2. A medium refresh step on day 3 was performed using a new culture dish with pre-equilibrated Blastocyst medium (Cook, Ireland) where embryos were placed. Actually Eeva™ culture dishes have 12 microwells under a single drop of medium: each well is identified by a letter (A, B, C, D) and a number (1, 2, 3): on day 3, embryos were moved from one dish to the other keeping the same microwell order (A1 to A1 in the new dish, etc.): This allowed to track individual embryo development and accurately correlate Eeva™ score on day 3 with embryo assessment on day 5. Dark field images were acquired every 5 min from the time of culture start until ET, cryopreservation or discharge; a video frame corresponded to 5 min in culture. All embryos were likewise assessed by bright field microscopy on day 3 to manually insert blastomere number, allowing calculating Eeva™ score prior to ET. Eeva™ algorhythm generated a calculation of High or Low probability of blastocyst formation based on kinetic growth parameters: the P2 value (time between first and second mitosis; P2 "high" range: 9.33–11.47 h) and the P3 value (time between second and third mitosis; P3 "high" range: 0.00–1.73 h). After morphology plus Eeva™-based embryo selection, either two embryos with "high" probability of blastocyst formation (DET) or one blastocyst on day 5 (SET) were transferred. In case of day 5 ET, the transferred embryo was chosen taking into account both the Eeva™ indication and the blastocyst morphological score; in detail, Eeva™ rating was used to chose between blastocysts with a similar morphological score.

The patients included in the study belonged to four groups: (a) those receiving day 3 DET with embryos selected by IMCS (DET-3 M group, $n = 106$, receiving 212 embryos), (b) those receiving day 3 DET with embryos selected by morphology plus Eeva™ (DET-3 ME group, $n = 48$, receiving 96 embryos), (c) those receiving day 5 SET with a blastocyst selected by conventional morphology (IMCS and blastocyst score) (SET-5 M group, $n = 126$, receiving 126 embryos), and (d) those receiving day 5 SET with a blastocyst selected by morphology plus Eeva™ (SET-5 ME group, n = 48, receiving 48 embryos).

Statistical analysis

Comparison among groups was performed using SAS (SAS Institute, Cary, NC) software package, using the unpaired t-test for continuous variables (shown as mean ± SD) and the Fisher exact test for categorical variables (shown as percentage). All statistical tests were two-sided and a P value of 0.05 or less was considered statistically significant.

Results

Among a total number of 1178 couples that completed IVF cycle in the study time period, 356 matched the inclusion criteria, and 328 of them authorized the inclusion of their data in the analysis. The clinical characteristics of the enrolled patients and the outcome of their IVF cycles are summarized in Tables 1 and 2, respectively.

All ETs were performed by experienced operators (AR, AC, GG, LDP) using the soft catheter Sydney Guardia (Cook, Australia) under transvaginal ultrasound guidance,

Table 1 Clinical data of enrolled patients according to the embryo selection method

	All (*n* = 328)	DET-3 M (*n* = 106)	DET-3 ME (n = 48)	SET-5 M (n = 126)	SET-5 ME (n = 48)	p
Age (years)	34.6 ± 3.0	34.7 ± 3.4	34.8 ± 3.0	34.2 ± 2.9	34.9 ± 1.9	*ns*
BMI (kg/m^2)	23.6 ± 3.9	23.1 ± 3.6	23.2 ± 4.2	24.1 ± 4.2	24.6 ± 3.5	*ns*
Day 3 FSH (IU/l)	6.9 ± 1.5	7.1 ± 1.7	6.7 ± 1.3	6.7 ± 1.3	6.9 ± 1.8	*ns*
AMH (ng/ml)	3.7 ± 2.5	3.3 ± 1.9	3.0 ± 1.8	4.6 ± 3.2	3.6 ± 2.0	*ns*
AFC	15.4 ± 7.3	14.3 ± 6.0	15.4 ± 9.4	16.9 ± 8.2	15.2 ± 5.5	*ns*
Total exogenous FSH (IU)	2083.7 ± 823.7	2245.2 ± 940.7	2112.0 ± 841.0	1902.2 ± 671.2	2026.2 ± 736.7	*ns*
Peak E2 (pg/ml)	2437.2 ± 1097.9	1933.9 ± 867	1672.9 ± 1178.3	2211.3 ± 1171.1	2436.3 ± 1317.3	*ns*
OSI	6.6 ± 3.8	5.3 ± 3.0	5.5 ± 3.6	7.9 ± 3.6	8.3 ± 5.2	*< 0.05**
Endometrial thickness (mm)	10.4 ± 2.0	10.2 ± 1.8	10.0 ± 2.2	10.7 ± 2.2	10.6 ± 1.7	*ns*
Retrieved oocytes	11.6 ± 4.0	10.2 ± 3.0	9.5 ± 2.6	13.4 ± 4.2	13.8 ± 4.9	*< 0.05**
Mature (MII) oocytes (%)	83.5 ± 15.2	85.6 ± 12.6	78.1 ± 19.5	81.8 ± 15.6	89.1 ± 12.6	*ns*
Insemination technique (%)						*ns*
IVF	24 (40/164)	26 (14/53)	25 (6/24)	25 (16/63)	17 (4/24)	
ICSI	76 (124/164)	74 (39/53)	75 (18/24)	75 (47/63)	83 (20/24)	
Fertilized (2PN) oocytes (%)	74.4 ± 17.6	69.8 ± 18.6	72.8 ± 18.3	79.7 ± 15.2	76.7 ± 16.6	*ns*
Cleaved embryos (%)	97.3 ± 7.9	96.8 ± 7.7	96.3 ± 11.6	98.0 ± 6.3	98.2 ± 7.1	*ns*

DET-3 M = patients receiving two day 3 embryos selected by IMCS; DET-3 ME = patients receiving two day 3 embryos selected by standard morphology plus Eeva™ score; SET-5 M = patients receiving one day 5 blastocyst selected by IMCS plus blastocyst morphological score; SET-5 ME = patients receiving one day 5 blastocyst selected by blastocyst morphological score plus Eeva™. AFC = antral follicle count. OSI = ovarian sensitivity index (retrieved oocytes × 1000 / total FSH dose). Data are expressed as mean ± SD or as absolute value and percentage. * Significance is referred to the comparison of both SET subgroups (SET-5 M and SET-5 ME) vs. both DET subgroups (DET-3 M and DET-3 ME)

applying the method that was previously published by our group [13]. No ET resulted to be difficult or forced to change catheter and to repeat the procedure.

Both subgroups of patients who received SET on day 5 had significantly more retrieved oocytes and higher ovarian responsiveness to COS (ratio between total FSH dose and retrieved oocytes, OSI) than the two subgroups that had DET on day 3 (Table 1). This is due to the fact that blastocyst transfer was performed only in patients with at least four good scored embryos on day 3, whereas all others patients received DET on day 3.

Overall, 1818 embryos were obtained in 328 IVF cycles; 482 embryos were transferred in uteri, and 193 implanted (overall implantation rate: 40%) originating 161 US-verified clinical pregnancies (overall clinical pregnancy rate: 49.1%). Thirty-two pregnancies were twin pregnancies, leading to an overall twinning rate of 19.8%, which is close to the average IVF twinning rate in Italy; however, single blastocyst transfer on day 5 never originated a twin pregnancy, whereas DET on day 3 obtained a very high twinning rate (33.3% in the DET-3 M group, 59.2% in the DET-3 ME group) (Table 2).

The clinical pregnancy rate was comparable in the four subgroups, with slight differences (Table 2). The implantation rate observed in DET-3 ME group was significantly higher than the one of DET-3 M group (44.8% vs. 30.2%, $p < 0.02$), whereas it was comparable to those of groups SET-5 M and SET-5 ME (Table 2). Eighteen

Table 2 Clinical outcome of IVF according to the embryo selection method

	All (n = 328)	Day 3 M (n = 106)	Day 3E (n = 48)	Day 5MM (n = 126)	Day 5 EM (n = 48)	p
Transferred embryos	482	212	96	126	48	
Clinical pregnancy rate (%)	49.1 (161/328)	45.3 (48/106)	56.2 (27/48)	49.2 (62/126)	50.0 (24/48)	*ns*
Implantation rate (%)	40.0 (193/482)	30.2 (64/212)	44.8 (43/96)	49.2 (62/126)	50.0 (24/48)	*< 0.02**
Twin pregnancy rate (%)	19.8 (32/161)	33.3 (16/48)	59.2 (16/27)	0/62	0/24	
Ongoing pregnancy rate (%)	43.6 (143/328)	39.6 (42/106)	50.0 (24/48)	43.6 (55/126)	45.8 (22/48)	*ns*

DET-3 M = patients receiving two day 3 embryos selected by IMCS; DET-3 ME = patients receiving two day 3 embryos selected by standard morphology plus Eeva™ score; SET-5 M = patients receiving one day 5 blastocyst selected by IMCS plus blastocyst morphological score; SET-5 ME = patients receiving one day 5 blastocyst selected by blastocyst morphological score plus Eeva™. Data are expressed as absolute value and percentage. * Significance is referred to the comparison of DET-3 M vs. DET-3 ME, SET-5 M and SET-5 ME

pregnancies underwent a spontaneous miscarriage in the first trimester, and finally the ongoing pregnancy rate at 12 weeks gestational age was similar in the four subgroups, without any significant difference (Table 2).

Discussion

The selection of embryos having the highest competence for pregnancy and live birth has been based for years on a single observation by inverted light microscopy performed on day 2, 3 or 5 of embryo culture. Repeated observations, although likely to give better insights about embryo competence, were used with caution because even a short exposure of embryos to suboptimal conditions outside the controlled environment of the incubator was thought to potentially affect the implantation potential.

The introduction of time-lapse technology (TLT) into the clinical practice has allowed providing a continuous surveillance of embryo growth, while maintaining stable culture conditions; moreover, the recording of previously unknown kinetic parameters of embryo development has provided new embryo-related variables available for analysis [8]. TLT was claimed to have the potential to improve embryo selection capability and, as a consequence, IVF outcome; indeed some studies reported higher pregnancy rates using TLT (reviewed in [12]), but others could not confirm this finding as they failed to observe any improvement of IVF results vs. the standard morphological embryo selection [5, 9, 10].

So far, none of the studies comparing TLT to the single observation, morphological embryo assessment used the evidence-based score named IMCS as a reference [6]. The difference between IMCS and the other scoring methods is that IMCS was constructed comparing the morphology of surely implanted embryos (dizygotic twin pregnancies after DET) vs. surely non-implanted embryos (no pregnancy after DET), and was therefore based on the evidence of implantation and clinical pregnancy. Indeed IMCS is the morphological score that was incorporated into a complex prediction model for IVF outcome, recently shown to predict live birth with a remarkably good precision [14]. Actually the present study is the first comparing embryo selection performed by an evidence-based morphological score vs. TLT.

In our study we aimed at assessing the impact of the adjunctive use of the Early Embryo Viability Assessment (Eeva™), an algorhythm for automatic embryo scoring at the cleavage stage, on the embryo selection process performed using conventional morphology. The possibility to predict the development of embryos observed in the first days of growth to the blastocyst stage, a concept underlying Eeva™ test, was previously demonstrated [3, 11, 17], and Eeva™ was found to be more reliable than a panel of embryologists with diverse experience in assessing embryo potential to evolve to blastocyst [4]. The clinical application of Eeva™ was already tested in a few studies, but conflicting results were obtained: an improved possibility of identification of cleaving embryos prone to reach the blastocyst stage was shown in a multicenter study [2], a positive effect of Eeva™ application on implantation and pregnancy rates was reported in other studies [1, 16], but some authors failed to observe any significant difference in pregnancy rates when Eeva™ was used in association with standard morphology vs. morphology alone performed on day 3 [9] or day 5 [7, 18].

We performed the present analysis on a very homogeneous patients' population, that was selected using very strict inclusion criteria; this strategy allowed obtaining four groups of patients with very similar clinical characteristics. The potentially confounding variable of the day chosen for ET (day 3 vs. day 5) was accounted for including in the study a subgroup in which Eeva™ was performed on day 3, but a single embryo was transferred on day 5, after a selection process that considered together Eeva™ results and blastocyst morphology.

Overall, we observed that when two embryos were selected using morphology plus Eeva™ and transferred on day 3 (DET-3 ME group), the implantation rate was significantly higher than when the IMCS alone was used (DET-3 M group); interestingly enough, the implantation rate of Eeva™-selected embryos on day 3 was comparable to the one of blastocysts morphologically selected and transferred on day 5 (SET-5 M and SET-5 ME groups). On one side this led to an unacceptably high twinning rate in the DET-3 ME group - suggesting that when Eeva™ is used to select embryos, only one embryo should be transferred in uteri – on the other side it demonstrated that Eeva™ has a remarkable efficacy in identifying which day 3 embryos have the best chance of development to blastocyst and implantation.

The positive effect of Eeva™ addition to classical morphology on the accuracy of embryo selection, however, was lost when embryo culture was prolonged to day 5; in fact, comparable implantation rates were obtained after single blastocyst transfer regardless Eeva™ was considered or not to chose the embryo to transfer. To this purpose it should be remarked that Eeva™ was constructed for use on day 3, and probably considering its results for the blastocyst selection process on day 5 represents an improper use.

Despite the described differences in the implantation rate, in our study both the clinical pregnancy rate and the ongoing pregnancy rate at 12 weeks were not significantly different in the four groups. This may be likely due to the relative low number of observations in some groups, but it may be noticed that even other authors reported that Eeva™ was ineffective in increasing the ongoing pregnancy rate when compared to a standard morphological score [7, 9]. Also a study showing an

increased pregnancy rate with Eeva™, unfortunately did not report about the ongoing pregnancy rate [1].

A limitation of our study, besides its retrospective nature, was that embryos were cultured in different incubators (Eeva™ vs. low oxygen tension incubators), culture dishes and volumes, and the influence of the culture conditions could not be clearly distinguished from the effect of embryo selection strategy. This confounder could not be eliminated because Eeva™ system cannot be housed in any incubator, but requires a specific model.

We are aware that our results may not be considered conclusive and should be verified on a larger scale and/or in properly weighted prospective trials. However, with all the above limitations, our findings suggest that Eeva™ algorhythm could be useful in improving the embryo selection accuracy of standard morphology when ET on day 3 is scheduled. Further, Eeva™ could be quite useful when applied to perform a SET on day 3, as according to our findings it could obtain clinical results comparable to the more time-demanding SET on day 5, after blastocyst culture.

Abbreviations

AFC: antral follicle count; AMH: anti-Mullerian hormone; BMI: body mass index; COS: controlled ovarian stimulation; DET: double embryo transfer; Eeva™: Early Embryo Viability Assessment; ET: embryo transfer; IMCS: Integrated Morphology Cleavage Score; OPU: US-guided oocyte retrieval (ovum pick-up); SET: single embryo transfer; TLT: Time-lapse embryo culture technology

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Authors' contributions

RA, CA, DPL and GG revised all the material, included the patients in the study, and analyzed the clinical data. CS and PC analyzed all laboratory data. FC performed the statistical analysis. BC performed critical revision of the manuscript. All authors contributed to write the manuscript, read the final version and approved it.

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Availability of data and materials

The study is a retrospective analysis of IVF cycles performed at our IVF Unit between March 2016 and July 2018. Data are stored in our institutional archive.

Ethics approval and consent to participate

The study was authorized as an observational study by the local Ethical Committee.

Consent for publication

A signed, written consent was retrospectively obtained from all patients accepting their data being included in the analysis.

Competing interests

The authors declare they have no competing interests.

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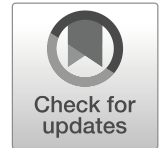

Morphokinetic analysis of cleavage stage embryos and assessment of specific gene expression in cumulus cells independently predict human embryo development to expanded blastocyst: a preliminary study

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Abstract
To assess whether morphokinetic features at the cleavage stage together with specific gene expression in cumulus cells (CCs) may be used to predict whether human embryos are able to achieve the expanded blastocyst stage on day 5. Eighty-one embryos were cultured using the Geri plus® time-lapse system. Twenty-seven embryos progressing to the expanded blastocyst stage (BL group) were compared with thirty-five embryos showing developmental arrest (AR group) and nineteen reaching the stage of early or not fully expanded blastocyst (nBL group). The analyzed morphokinetic variables were pronuclear appearance (tPNa), pronuclear fading (tPNf), and completion of cleavage to two, three, four, and eight cells (t2, t3, t4, and t8). CCs were analyzed by RT-qPCR for bone morphogenetic protein 15 (*BMP15*), cytochrome *c* oxidase subunit II (*COXII*), ATP synthase subunit 6 (*MT-ATP6*), connexin 43 (*Cx43*), and heme oxygenase-1 (*HO-1*). Embryos of BL group showed a significantly faster kinetic. *BMP15*, *COXII*, and *MT-ATP6* mRNA expression was significantly higher in CCs of BL group embryos, whereas *Cx43* and *HO-1* mRNA levels were higher in AR group. Kinetic parameters and gene expression were not significantly different between either the BL and nBL groups or the AR and nBL groups. ROC curves showed that the most predictive cut-offs were t2 < 26.25 for morphokinetics and *COXII* > 0.3 for gene expression. Multivariable logistic regression analysis showed that morphokinetic variables and gene expression were both valuable, independent predictors of embryo development to expanded blastocyst. Our results suggest the possibility of developing integrated prediction models for early embryo selection at the cleavage stage.

Keywords Time-lapse · Embryo morphology · Cumulus cells · Gene expression · Blastocyst · Human IVF

Introduction

In the last decades, elective single blastocyst transfer progressively became a widespread clinical strategy to reach a fairly good pregnancy rate with very low risk of twin pregnancy [1]. However, extensively applied embryo culture at the blastocyst stage is time- and work-demanding for the in vitro fertilization (IVF) laboratories, and, furthermore, some data raise the suspect that prolonged culture could increase epigenetic alterations in the embryo [2]. An alternative strategy would be transferring in utero a single embryo, at cleavage stage, having the highest chance to reach the blastocyst stage, instead of a single blastocyst. The problem, however, would be how to select, among cleaving embryos, those with the best probability to progress to the blastocyst stage. Some morphological scoring criteria applicable in vitro on day three were proposed, but none of them proved to be fully reliable in predicting embryo further development and competence [3].

In the more recent last years, the morphokinetic evaluation using time-lapse systems (TLS) joined conventional morphological evaluation providing a new tool to identify markers of

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embryo competence and development potential [4]. Indeed, the use of TLS offers a continuous, dynamic, individualized assessment of embryo growth: information on the timing of specific cleavage events could be used to develop algorithms aimed at improving early embryo selection. The Early Embryo Viability Assessment (Eeva™) software is an example of algorithm that applies embryo growth parameters to identify on day three the embryos with the highest probability to become blastocyst [5–7].

An alternative, non-invasive approach to precociously estimate embryo competence is inspired by the concept that the follicular microenvironment can significantly affect the quality of the oocyte and, as a consequence, of the embryo deriving from each specific oocyte [8]. Within the study of the follicular microenvironment biomarkers, attention was initially given to the follicular fluid (FF) biochemical composition, whereas the study of specific gene expression in the cumulus cells (CCs), potentially affecting embryo development, became progressively more popular in the last years [9, 10].

The quality of CCs can be influenced both by some oocyte-secreted factors and by the intrinsic characteristics of these cells. Indeed, the oocyte produces several growth factors, such as growth differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (*BMP15*), which are known to regulate growth, differentiation, and function of granulosa and theca cells during follicular development, and plays a fundamental role in oocyte maturation, ovulation, fertilization, and embryonic competence [11–14]. On the other hand, the expression of some structural proteins in CCs is of paramount importance for their nurturing function. For example, some connexins, such as connexin 43 (*Cx43*), are expressed in human CCs and were claimed to affect oocyte quality [15]; they also represent markers of follicle integrity in both humans and animal models [16]. Moreover, one aspect of CCs still scarcely investigated is the quality of their metabolism, which is likely to be involved in embryo development.

We previously demonstrated that a status of enhanced oxidative stress in CCs, arising when the reactive oxygen species (ROS) production overwhelms the intrinsic antioxidant defense, negatively affects oocyte competence [17, 18]; as the main source of ROS are mitochondria, it is evident that mitochondrial respiration must be strictly regulated and well-coupled to oxidative phosphorylation in order to sustain the energy requirements of CCs and avoid ROS production. Therefore, the analysis of genes involved in this function of CCs could be quite relevant.

In the attempt of finding a multivariable approach taking into account different issues which may be critical in oocyte maturation, we designed the present study aiming at (a) identifying morphokinetic features of cleaving embryos able to predict their timely development to the blastocyst stage, (b) assessing the expression of specific genes in CCs and understanding if they could affect the chance of embryo growth to blastocyst, and (c) checking whether morphokinetic variables and the expression of specific genes in CCs could be independent predictors of embryo development.

Materials and methods

Patients

The study considered 81 embryos obtained from 15 women (mean age: years, range 33–40) with normal body mass index (BMI 18–25), serum day 3 FSH < 12 IU/l, serum AMH > 1.2 ng/ml, and antral follicle count (AFC) > 8, who underwent intracytoplasmic sperm injection (ICSI) at our IVF Unit (Turin, Italy), to treat male-related infertility. Exclusion criteria were the following: presence of polycystic ovary syndrome or endometriosis and history of severe ovarian hyperstimulation syndrome. The patients' clinical characteristics and the outcome of controlled ovarian stimulation (COS) were recorded, including the total dose of exogenous FSH, the peak estradiol (E2) level, the number of retrieved oocytes, the ovarian sensitivity index (OSI = retrieved oocytes × 1000/total gonadotropin dose) [19], the fertilization rate, and the clinical (ultrasound visualization of the gestational sac) pregnancy rate (CPR) per embryo transfer (ET).

The study was carried out in accordance with the Declaration of Helsinki and was authorized as a prospective observational study by the local Ethical Committee. A signed, written informed consent was obtained from all patients.

Controlled ovarian stimulation, oocyte retrieval, and cumulus cells collection

The gonadotropin-releasing hormone (GnRH)-agonist "long" protocol with recombinant FSH (Gonal-F®, Merck, Germany) at individually tailored daily dose (100–300 IU s.c.) was used to carry out controlled ovarian stimulation (COS). Circulating E2 dosage assessment and transvaginal US examination were performed every second day from stimulation day 7 in order to monitor follicular growth. The dose of FSH was adjusted accordingly. A single s.c. injection of 10,000 IU hCG (Gonasi HP, IBSA, Switzerland) was administered to trigger ovulation, when at least two follicles reached 18 mm mean diameter, with appropriate E2 levels.

US-guided oocyte retrieval (OPU) was performed 35–37 h after hCG trigger under local anesthesia (paracervical block). Follicular fluid was aspirated and immediately observed under a stereomicroscope. Cumulus-oocyte complexes (COCs) were washed in buffered medium (Flushing medium, Cook Ltd., Ireland); 2 h after OPU, oocytes and CCs were separated from each individual COC by gently pipetting in 40-μl HEPES-buffered medium containing 80 IU/ml hyaluronidase (Synvitro Hyadase, Origio Medicult, Denmark) [20]. CCs

samples were separated from the corresponding mature oocytes and stored in liquid nitrogen until gene expression analysis.

Preparation of semen samples, ICSI, and fertilization check

Sperm concentration, motility, and morphology were assessed according to the World Health Organization guidelines (WHO laboratory manual 5th ed., 2010). Semen samples were prepared using density gradient centrifugation in order to select normally motile morphologically normal spermatozoa. ICSI was performed with all available mature oocytes within 2 h after CCs removal. Normal fertilization was confirmed when the presence of two pronuclei (2PN) and the extrusion of the second polar body were observed 16–18 h after injection.

Time-lapse embryo culture

Embryos were cultured using the Geri plus® TLS (Genea Biomed, Germany) with integrated embryo monitoring system in microwells (one zygote/microwell). The dish format allowed following each embryo individually even if all embryos shared a common 80-μl drop of medium. Embryos were cultured in pre-equilibrated Cleavage medium (Cook, Ireland) overlaid with mineral oil up to day 3; at this stage, a change of medium was performed and a new one (Blastocyst medium, Cook, Ireland) was added and kept until the blastocyst stage. Bright field images captured by the Geri plus® system were acquired every 5 min from the time of fertilization until the time of embryo transfer (ET), cryopreservation, or discharge.

Embryo morphological evaluation was firstly performed on day 2 using the Integrated Morphology Cleavage Score (IMCS) [21]; embryo selection for ET was performed on day 5 considering both conventional morphology [22] and morphokinetic parameters [4]. All the videos collected by TLS were analyzed, and the following morphokinetic parameters (times) were considered: pronuclear appearance (tPNa); pronuclear fading (tPNf); completion of cleavage to two, three, four, and eight cells (t2, t3, t4, and t8 respectively); and time intervals tPNf-tPNa, t2-tPNf, t3-t2, t4-t3, t4-t2, and t8-t4. Time intervals from t8 (completed cleavage to eight cells) to expanded blastocyst (t cavitation, t blastocyst, t hatching) were annotated, but not considered in our analysis as our aim was to identify early predictive biomarkers of embryo development.

Embryos that progressed to the expanded blastocyst stage on day 5 (score 3 according to [22]) were included in the blastocyst group (BL group; $n = 27$); embryos that progressed to the early or not fully expanded blastocyst stage on day 5 (score 1 or 2) were included in the not-expanded blastocyst group (nBL group; $n = 19$), whereas those undergoing developmental arrest, reaching as maximum the morula stage on day 5 and remaining at the same stage until day 6 were included in the arrested group (AR group; $n = 35$). Blastocyst assessment was performed at the same time interval (116 ± 2 h after injection) for all embryos. Thirteen expanded blastocysts were transferred in utero (one per patient) using the soft catheter Sydney Guardia (Cook, Australia) under transvaginal US guidance, applying the method previously published by our group [23].

RNA extraction, cDNA synthesis, and RT-qPCR

CCs corresponding to each embryo were analyzed by RT-qPCR. Cells-to-CT™ 1-Step PowerSYBR® Green Kit (Ambion; Life Technologies, Italy) was used to extract total RNA following the manufacturer's instruction. Twenty microliters of RNA were used for retrotranscription using SensiFAST™ cDNA Synthesis Kit (Bioline, Meridian Bioscience, UK) at 42 °C for 15 min and 85 °C for 5 min. cDNAs were stored at – 80 °C until PCR analysis. PCR primers were designed using NCBI/Primer-BLAST and synthesized by Sigma-Merck (UK): bone morphogenetic protein 15 (*BMP-15*), 5′-GGCTCCTAGGGCATTCACTG-3′, 5′-CCTCGGTTTGGTCTGAGAGG-3′ [24]; connexin 43 (*Cx43*), 5′-TACCAAACAGCAGCGGAGTT-3′, 5′-TGGGCACCACTCTTTTGCTT-3′ [25]; subunit II of cytochrome C oxidase (COX or respiratory complex IV) (*COXII*), 5-CGACTACGGCGGACTAATCT-3′, 5′-TCGATTGTCAACGTCAAGGA-3′ [26]; a mitochondrial gene coding for the ATP synthase subunit 6 (*MT-ATP6*), 5′ CCAATAGCCCTGGCCGTAC-3′, 5′ CGCTTCCAATTAGGTGCATGA-3′ [26]; heme oxygenase 1 (*HO-1*) [17], 5′-AGGAGGAGATTGAGCGCCAC-3′, 5′-TCTGGTCCTTGGTGTCATGG-3′; beta-2-microglobulin (*B2M*), 5′-AGCAAGGACTGGTCTTTCTATCTC-3′, 5′-ATGTCTCGATCCCACTTAACTA-3′ [26]; *S14 ribosomal* protein gene (*S14*), 5′-AGGTGCAAGGAGCTGGGTAT-3′, 5′-TCCAGGGGTCTTGGTCCTATT-3′ [27]. *B2M* and *S14* were used as endogenous controls as they were previously confirmed to be stable (M value of 0.176 and 0.183, respectively) in human CCs using the CFX Manager Software analysis [27].

SensiFAST™ SYBR® No-ROX Kit (Bioline, Aurogene Srl, Italy) was used to perform RT-qPCR following manufacturer's protocol: 1 cycle at 95 °C for 2 min, 40 cycles at 95 °C for 5 s, 1 cycle at 60 °C for 30 s and 1 cycle at 95 °C for 1 min. Gene expression analysis was performed by CFX Manager Software (BioRad, CA, USA). Each reaction was performed in triplicate. Melt curve analysis confirmed the specificity of PCRs.

Statistical analysis

Continuous variables are shown as mean ± standard deviation (SD), whereas categorical variables as absolute and relative

frequencies. At univariate analysis, the analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons or the non-parametric Kruskal-Wallis rank test, as appropriate, were carried out to compare the BL, the nBL, and the AR groups for all the recorded times and time intervals. The correlation between embryo morphokinetic parameters and CCs gene expression was assessed using the Spearman rank correlation coefficient.

Multivariable analysis models were then fitted to evaluate the independent effect of morphokinetic parameters and gene expression on blastocyst development: blastocyst development at day 5 (arrested embryos – AR group vs. expanded blastocyst – BL group) was set as the dependent variable of logistic regression models, in which different time-lapse parameters and gene expressions in CCs were taken as independent variables.

Significance level was set at $\alpha = 0.05$. All analyses were performed using STATA 14 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP).

GraphPad Prism V7 was used to generate the receiver operating characteristic (ROC) curves in order to assess the performance of different morphokinetic and gene expression variables in identifying embryos with blastocyst development potential. The area under the ROC curve (AUC) was determined to provide a numerical summary of the indicator's performance. Cut-off points were identified for specificity and sensitivity when the Youden's Index (sensitivity + specificity − 1) was maximal, considering BL and AR as test and control groups, respectively.

Results

Patients

Table 1 summarizes the clinical characteristics of the patients and the outcome of their IVF cycles. The age and biomarkers were as expected of good prognosis patients; indeed, the retrieved oocytes mean number was 9.3 ± 2.3, and the clinical PR/ET was 61.5%, in line with the predicted outcome after transfer in utero of a single expanded blastocyst in such patients.

Morphokinetic analysis

Overall, 81 embryos were obtained, and during in vitro culture in the TLS GERI plus®, 27 embryos progressed to the expanded blastocyst stage (BL group), 35 embryos arrested their development on day 5 and remaining at the same stage until day 6 (AR group), whereas 19 embryos reached an intermediate state of development (early blastocyst or not fully expanded blastocyst) on day 5 (nBL group). Interestingly enough, the morphological score IMCS [21] on day 2 was comparable in the three groups (9 ± 4.6 vs. 7.3 ± 1.4 vs. 8.8 ± 4.3 for BL, nBL, and AR groups, respectively; $p > 0.05$).

Table 1 Patients' clinical characteristics and IVF outcome

Age (years)	35.8 ± 2.9
BMI (kg/m^2)	23.3 ± 3.2
Basal (day 3) FSH (IU/l)	7.5 ± 2.8
AMH (ng/ml)	3.4 ± 2
AFC (*n*)	16 ± 8.6
Total exogenous FSH (IU)	2254.2 ± 734.8
Peak E2 (pg/ml)	1823.3 ± 486.8
Endometrial thickness (mm)	11 ± 1.6
Retrieved oocytes (*n*)	9.3 ± 2.3
OSI (*n*)	4.5 ± 1.7
Maturation rate (%)	90 ± 14.3
Fertilization rate (%)	81.7 ± 16.2
Cleavage rate (%)	97.4 ± 6.3
Blastocyst rate (%)	61.3 ± 28.4
Implantation rate % (*n*)	61.5 (8/13)
Clinical PR/ET % (*n*)	61.5 (8/13)
Live birth rate/ET % (*n*)	61.5 (8/13)

Data are shown as mean ± standard deviation, with the only exception of the last variables which are expressed as a percentage

BMI body mass index, *AFC* antral follicle count, *FSH* follicle-stimulating hormone, *E2* estradiol, *OSI* ovarian sensitivity index = number of retrieved oocytes × 1000/total FSH dose

Comparing the three groups, we found that times tPNa, tPNf, t2, t3, t4, and t2-tPNf showed significant differences (Table 2): embryos of the BL group showed a significantly quicker early kinetic than embryos in the AR group ($p < 0.05$) (Fig. 1). We also observed a shorter (although not significantly) t8 time in the BL group.

The interval t2-tPNf, corresponding to the first cleavage, was significantly shorter in the BL group ($p = 0.035$), and the delay of embryos in AR group vs. those in BL group started as soon as pronuclei appeared (tPNa), progressively increasing until t8 as shown by longer Δtime value (Table 2 and Fig. 1). No significant differences were observed comparing BL vs. nBL group, or nBL vs. AR group (data not shown).

Gene expression in CCs

CCs corresponding to the 81 embryos assessed by TLS GERI plus® were analyzed by RT-qPCR in order to assess the expression of genes *BMP15*, *Cx43*, *COXII*, *MT-ATP6*, and *HO-1*. We observed that *BMP15*, *COXII*, and *MT-ATP6* mRNA expression was significantly lower ($p < 0.05$ or $p < 0.01$) in CCs corresponding to embryos of the AR group than in those corresponding to embryos of the BL group (Fig. 2). On the contrary, *Cx43* and *HO-1* mRNA expression was significantly

Table 2 Morphokinetic parameters recorded by TLS GERI plus®

	BL group (*n* = 27)	nBL group (*n* = 19)	AR group (*n* = 35)	*p* value*	Δ (AR group - BL group) time	Δ (nBL group - BL group) time	Δ (AR group - n BL group) time
tPNa (h)	6.6 ± 1.5	7.0 ± 1.4	7.5 ± 1.5	*0.0496^*	0.9	0.4	0.5
tPNf (h)	20.9 ± 2.2	22.2 ± 2.4	24.0 ± 3.5	*0.0006*	3.1	1.3	1.8
t2 (h)	23.7 ± 1.9	24.7 ± 2.4	26.5 ± 3.6	*0.0028*	2.8	1.0	1.8
t3 (h)	33.1 ± 4.0	34.9 ± 3.4	36.9 ± 6.0	*0.0031*	3.8	1.8	2.0
t4 (h)	36.0 ± 4.8	37.6 ± 3.8	41.2 ± 8.7	*0.0007*	5.2	1.6	3.6
t8 (h)	57.4 ± 7.7	62.8 ± 15.2	65.0 ± 12.7	0.0984	7.6	5.4	2.2
tPNf-tPNa (h)	14.6 ± 2.4	15.3 ± 1.8	16.3 ± 3.5	0.1485	1.7	0.7	1.0
t2-tPNf (h)	2.5 ± 0.4	2.5 ± 0.4	2.8 ± 0.7	*0.0368*	0.3	0	0.3
t3-t2 (h)	9.4 ± 3.6	10.2 ± 3.1	10.4 ± 4.6	0.1310	1.0	0.8	0.2
t4-t2 (h)	12.8 ± 3.8	12.9 ± 3.4	14.2 ± 7.9	0.3479	1.4	0.1	1.3
t8-t4 (h)	21.9 ± 7.6	28.3 ± 17.5	26.4 ± 10.2	0.3557	4.5	6.4	-1.9

Times and time intervals are shown for embryos reaching the expanded blastocyst stage (BL group), the not-expanded blastocyst group (nBL group) and those whose development arrested (AR group). Values are expressed as mean ± standard deviation. In the ΔTime column, the difference between embryos in groups AR vs. BL, nBL vs. BL and AR vs. nBL were calculated for each parameter. The *p*-value column shows the significance of the overall comparison among the three groups, performed with analysis of variance (^) or Kruskal-Wallis rank test, as appropriate

Italic *p* values indicate significant differences

higher in CCs corresponding to embryos of the AR group ($p < 0.001$) (Fig. 2). Instead, no significantly different gene expression was observed comparing nBL vs. BL group or nBL vs. AR group (Fig. 2). The observed difference between AR and BL groups was confirmed also by the analysis of gene expression intra-patient variability: within each patient-specific cohort of zygotes, most CC samples of the BL group clustered above the median for *BMP15*, *COXII*, and *MT-ATP6*, and below the median for *Cx43* and *HO-1* mRNA expression (Fig. 3). The opposite trend was observed for the AR group.

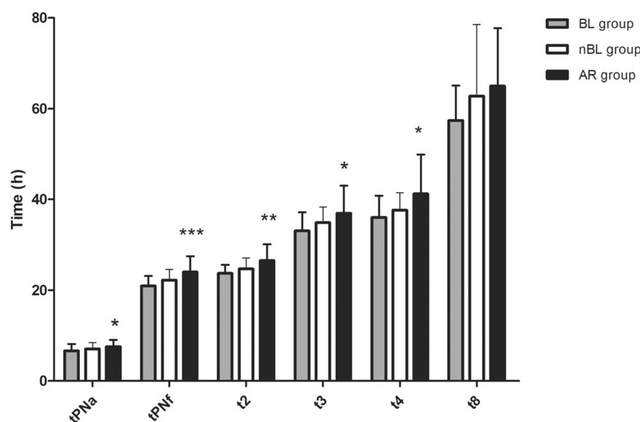

Fig. 1 Morphokinetic of embryos reaching the expanded blastocyst stage (BL group), the not-expanded blastocyst stage (nBL group) and of those whose development arrested (AR group). Times legend is the following: pronuclear appearance (tPNa), pronuclear fading (tPNf), completion of cleavage to two, three, four, and eight cells (t2, t3, t4, and t8 respectively). Values are expressed as mean ± standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ AR group vs. BL group

Correlation between gene expression in CCs and morphokinetic parameters

Overall, we observed a negative relationship between mRNA levels of the analyzed genes and the evaluated morphokinetic parameters. As shown in Table 3, embryos developing slower had a lower expression of *BMP15*, *COXII*, and *MT-ATP6* in the corresponding CCs. In fact, we found a statistically significant negative correlation between *BMP15* mRNA levels in CCS and the times t3 (rho = − 0.34, $p < 0.05$), t4 (rho = − 0.39, $p < 0.05$), t8 (rho = − 0.40, $p < 0.05$), time intervals tPNf-tPNa (rho = − 0.33, $p < 0.05$) and t8-t4 (rho = − 0.32, $p < 0.05$) (Table 3).

Logistic regression models

At multivariable analysis, performed only on the AR and BL groups, the expression of the two CCs genes *BMP15* and *COXII* was independently and significantly associated with a higher probability of development to expanded blastocyst on day 5 ($p < 0.05$) (Table 4). On the contrary, *HO-1* mRNA level in CCs corresponded to a higher risk of embryo developmental arrest, although not statistically significant (not shown).

Predictive cut-off values of embryo development on day 5

ROC curve analysis was applied to identify optimal cut-off values of both morphokinetic parameters and CCs gene expression predictive of development to expanded

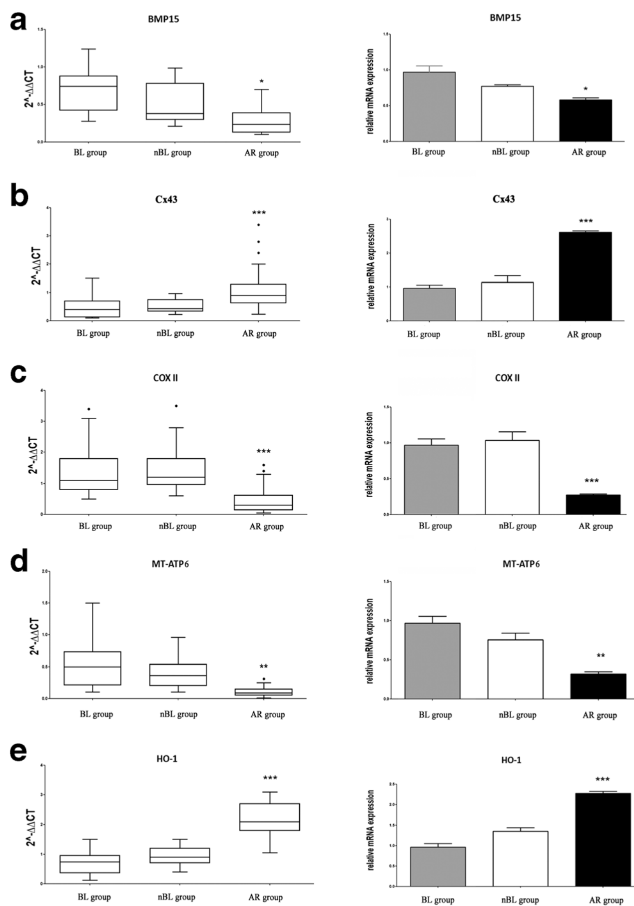

Fig. 2 Gene expression of *BMP15*, *Cx43*, *COXII*, *MT-ATP6*, and *HO-1* in the CCs of the three analyzed groups. For each gene, the box-plots of 2^{-ddCT} data (on the left) and the relative mRNA expression (on the right) analyzed by RT-qPCR in individual cumulus cells corresponding to embryos reaching the expanded blastocyst stage (BL group, $n = 27$), not expanded blastocyst stage (nBL group, $n = 19$), or those whose development arrested (AR group, $n = 35$) are shown. Measurements of bone morphogenetic protein 15 (*BMP15*), cytochrome *c* oxidase subunit II (*COXII*), ATP synthase subunit 6 (*MT-ATP6*), connexin 43 (*Cx43*), and heme oxygenase-1 (*HO-1*) were performed in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ AR group vs. BL group

blastocyst on day 5. According to maximization of the Youden Index, the parameters showing an optimal cut-off value were t2 for morphokinetic assessment (cut-off at t2 < 26.25 having sensitivity = 96 % and specificity = 47 %, AUC = 0.75; 95 % CI, 0.62–0.87; $p = 0.001$) (Fig. 4a), and *COXII* for mRNA expression (cut-off at COXII > 0.3 having sensitivity = 92 %, specificity = 75%, AUC = 0.86; 95 CI, 0.72–0.99; $p = 0.001$) (Fig. 4b).

Discussion

The identification of markers able to accurately predict embryo development to the expanded blastocyst stage by studying the early stages of embryo growth (within 72 h from fertilization) is of high practical interest for IVF Labs. In fact, it

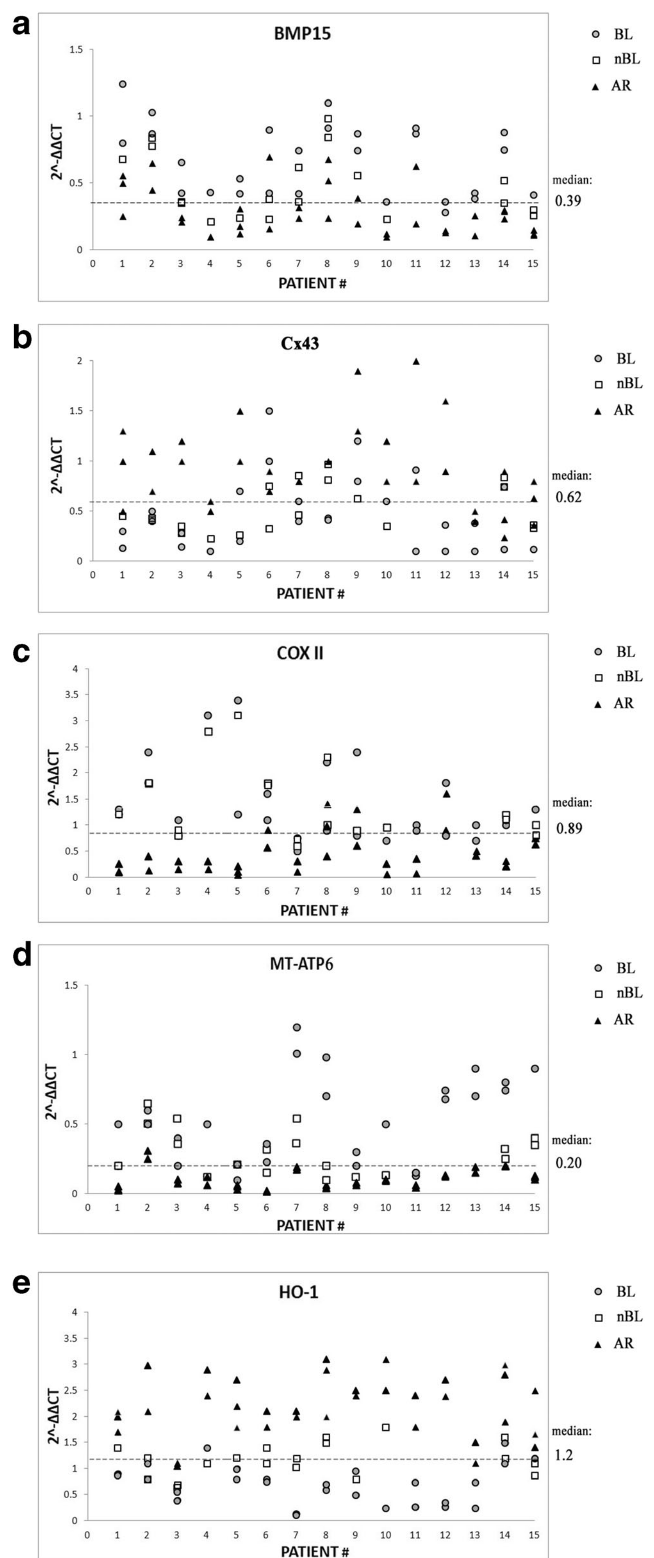

Fig. 3 Intra-patient variability analysis for the expression of *BMP15*, *Cx43*, *COXII, MT-ATP6*, and *HO-1*. For all analyzed genes, the 2^{-ddCT} data of each cumulus cell (CC) sample are grouped by patient and are represented according to embryo development (expanded blastocyst = gray circles, not-expanded blastocyst = white squares, arrested development = black triangles). The median general expression value has been reported for each gene as a dashed black line

Table 3 Correlation between the expression of specific genes in the CC and embryo morphokinetic parameters

	tPNa	tPNf	t2	t3	t4	t8	tPNf-tPNa	t2-tPNf	t3-t2	t4-t2	t8-t4
BMP15	− 0.0625	− 0.2921	− 0.2960	− 0.3352	− 0.3885	− 0.3950	− 0.3258	− 0.2946	− 0.2510	− 0.1960	− 0.3160
p value	0.7014	0.0674	0.0637	0.0345*	0.0132*	0.0117*	0.0402*	0.0650	0.1183	0.2255	0.0470*
Cx43	0.0760	− 0.2700	− 0.1610	− 0.1255	− 0.1067	0.0644	− 0.3578	0.4260	− 0.0359	0.0142	0.1520
p value	0.6412	0.0920	0.3209	0.4405	0.5121	0.6928	0.0234	0.0061	0.8258	0.9305	0.3490
COXII	0.0029	− 0.1443	− 0.2216	− 0.3407	− 0.1485	− 0.1339	− 0.2702	− 0.3664	− 0.0938	0.1354	− 0.1443
p value	0.9857	0.3745	0.1694	0.0315*	0.3604	0.4102	0.0918	0.0200*	0.5647	0.4048	0.3743
MT-ATP6	− 0.2800	− 0.4024	− 0.4657	− 0.5390	− 0.3334	− 0.0377	− 0.3594	− 0.3995	− 0.1903	0.1465	0.1193
p value	0.0801	0.0100 **	0.0025**	0.0003***	0.0355 *	0.8172	0.0227 *	0.0107 *	0.2396	0.3672	0.4634
HO-1	0.1261	− 0.0981	− 0.0029	− 0.0213	0.0013	0.0443	− 0.2178	0.1242	0.0533	0.0156	0.0599
p value	0.4380	0.5469	0.9860	0.8964	0.9938	0.7860	0.1770	0.4452	0.7441	0.9239	0.7136

Spearman rank coefficients and p-values for correlations of mRNA of bone morphogenetic protein 15 (*BMP15*), cytochrome c oxidase subunit II (*COXII*), ATP synthase subunit 6 (*MT-ATP6*), connexin 43 (*Cx43*) and heme oxygenase-1 (*HO-1*) with timelapse parameters (pronuclear appearance (tPNa), pronuclear fading (tPNf), completion of cleavage to two, three, four and eight cells (t2, t3, t4, and t8 respectively), time intervals tPNf-tPNa, t2-tPNf, t3-t2, t4-t3, t4-t2 and t8-t4) are shown: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$

could allow substituting the selective transfer of a single blastocyst with that of a single day 3 embryo having an optimal potential to become a fully developed, expanded blastocyst on day 5. In practical terms, this would reduce the risk of epigenetic alterations due to a more prolonged embryo permanence in artificial environment [2].

Table 4 Multivariable logistic regression analysis

	OR	CI 95%	*p*
tPNa	1.86	0.97; 3.6	0.063
BMP15	0.18	0.04; 0.88	0.034
tPNf	1.25	0.90; 1.71	0.175
BMP15	0.22	0.05; 1.01	0.052
t3	1.04	0.86; 1.25	0.696
BMP15	0.19	0.04; 0.96	0.045
t4	1.12	0.94; 1.33	0.202
BMP15	0.17	0.03; 0.96	0.046
dPN	1.1	0.72; 1.65	0.685
COXII	0.03	0.003; 0.30	0.003
t2	1.3	0.82; 2.02	0.282
COXII	0.03	0.002; 0.3	0.004
t3	1.05	0.85; 1.30	0.632
COXII	0.03	0.003; 0.32	0.004
t4	1.38	0.88; 2.13	0.157
COXII	0.02	0.001; 0.32	0.005
t8	1.40	0.99; 1.90	0.053
COXII	0.01	0.003; 0.41	0.014

Independent effect of bone morphogenetic protein 15 (*BMP15*) and cytochrome c oxidase subunit II (*COXII*) genes expression in cumulus cells and morphokinetic parameters with respect to the probability of embryo development on day 5 (arrested group vs. blastocyst group). Odds ratio (OR), 95% Confidence Interval (CI 95%) and *p*-value are shown for each model

Unfortunately, none of the "classical" morphological scores of the cleaving embryo has proven to be fully reliable in identifying embryos with the best development capability [3]. The rather recent advent of TLS technology provided the possibility to obtain a noticeable bulk of data about the kinetic of human embryo growth in culture, allowing the evaluation of embryo features previously unknown after conventional morphology analysis. Despite this, TLS benefits on embryo selection and IVF outcome, claimed by some authors [28], are disputable according to others [29–33] and, even most important, are partially due to the unperturbed culture conditions rather than to the application of morphokinetic data. Artificial intelligence approach based on deep neural networks has recently been applied to TLS, and would hopefully be useful to develop novel embryo selection algorithms [34]; to date, however, the results of this approach are still unavailable in the clinical practice.

The possibility to use TLS to select the best embryo at the cleavage stage, without waiting day 5, is still hypothetical. Some authors claim that TLS technology may dissect specific events occurring during and soon after fertilization and may identify early putative markers of embryo quality [35]. Recently, our group showed that Eeva™, an algorithm based on the analysis of early embryo growth, is able to improve embryo selection accuracy of standard morphology by effectively predicting which embryo at day 3 will develop into a viable blastocyst on day 5 [36]. Today, however, a panel of specific early morphokinetic features predictive of a favorable embryo development to the blastocyst stage is still lacking. This study was aimed at tackling the issue, providing also a

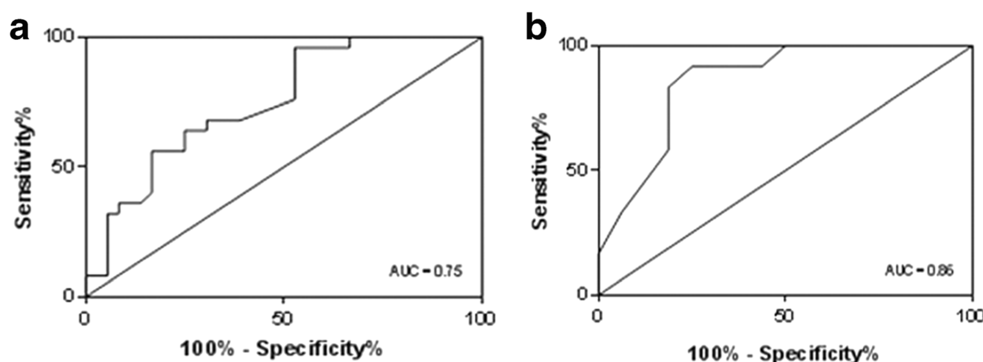

Fig. 4 ROC curves for the predictive potential of TLS time of completion of cleavage to two cells (t2) (a) and cytochrome *c* oxidase subunit II (*COXII*) gene expression (b) with respect to the probability of embryo development to expanded blastocyst on day 5. Cut-off values are at t2 < 26.25 (sensitivity = 96% and specificity = 47%, AUC = 0.75; 95% CI, 0.62–0.87; $p = 0.001$) and at *COXII* > 0.3 (sensitivity = 92%, specificity = 75%, AUC = 0.86; 95 CI, 0.72–0.99; $p = 0.001$)

link between embryo morphokinetics and specific molecular markers of gene activation in the corresponding CCs.

Using a last generation TLS, we observed that embryos able to achieve the expanded blastocyst stage on day 5 were characterized by a significantly faster cellular kinetic already during the first 3 days of development; interestingly, these embryos showed a quicker cleavage rate and then proceeded progressively increasing the time gap (ΔTime) relative to the embryos who arrested their development; in fact, the gap between embryos reaching the expanded blastocyst stage and those undergoing developmental arrest was maximal on day 3 (ΔTime t8 = 7.6 h). Embryos showing an optimal development on day 5 had a day 2 morphological score comparable to that of the embryos arresting their growth, but very precociously showed shorter and anticipated cleavage times, and kept being faster until day 3, when their DNA started to get its full activation.

The correlation between morphokinetic markers, embryo development, blastocyst formation rates, and ploidy has already reported [37]. Our observations confirm previous studies reporting that a timely blastocyst development on day 5 can be predicted quite early looking at the duration of the first cytokinesis, as well as at the time interval between the end of the first cleavage and the initiation of the second, the time interval between the second and third cleavage [38], the duration of the second [39] and third cleavage divisions [40], and the absence of direct cleavage from one to three cells [41]. Also, Cruz et al., analyzing the morphokinetic data of 834 embryos, found that blastocyst formation rate and blastocyst quality were related to the cleavage kinetic of early embryo [42]. Likewise, Dal Canto et al. showed that embryos that cleave earlier from the 2-cells to 8-cells stage have the highest potential to become blastocyst, expand, and implant [43]. Hashimoto et al. reported that the time intervals between the 3-cells and the 4-cells stage and between the 5-cells and the 8-cells stage were shorter for embryos with high-scored blastocysts compared to low-scored [40]. In addition, Minasi et al., analyzing 928 blastocysts morphokinetic parameters, found that euploid embryos needed shorter intervals to expand to blastocyst and hatch compared to aneuploid embryos [44], while Campbell et al. described that aneuploid embryos showed a delayed initiation of compaction and blastulation, as well as a longer time to reach the full blastocyst stage [45].

Looking at the clinical implications of embryo kinetics, it was reported that the transfer of fresh, slow-growing blastocysts resulted in lower implantation and clinical pregnancy rate compared to the transfer of fully expanded day 5 blastocysts [46, 47], probably due both to a reduced embryo competence and to a loss of embryonic-endometrial synchrony [48]. Moreover, the transfer of a fresh early blastocyst on day 5 was found to obtain success rates comparable to the transfer of a thawed expanded blastocyst cryopreserved on day 6 from the same cohort [49], suggesting that embryo kinetic is more important than the embryo developmental stage itself in determining IVF outcome. In fact, in cases where only early or not fully expanded blastocyst are available on day 5, extending culture until cryopreservation on day 6, and subsequent transfer after thawing may result in successful pregnancy. Taken together, these observations lead us to consider for our analysis the fully expanded blastocyst as the ideal developmental stage that most likely relates to higher embryo implantation. However, the transfer of slower-growing embryos may represent a feasible option in the absence of expanded blastocyst on day 5 or a favorable strategy to increase the cumulative pregnancy rates after frozen-thawed embryo transfer cycles. Using our combined approach, embryos that will develop into early blastocysts cannot be distinguished from those that will arrest, probably due to the limited sample size; however, we observed that embryos achieving the stage of early or not fully expanded blastocyst showed a morphokinetic behavior similar to embryos that reached full expansion on day 5 of development and a quicker, although not significant, kinetics compared to embryos undergoing developmental arrest.

Gene expression in CCs was already proposed as a non-invasive assay useful to predict embryo development and pregnancy [8]. An increased expression of a specific set of genes in CCs was previously related to a higher likelihood of oocyte fertilization, embryo quality, and clinical pregnancy [20, 50–52].

In this study, assessing the gene expression related to oocyte-secreted factors in CCs, we showed that *BMP-15* was significantly more expressed, and *Cx43* significantly less expressed, in the CCs corresponding to embryos developing to expanded blastocyst on day 5 than in those corresponding to embryos with arrested development. The significant relationship between *BMP-15* expression in CCs and optimal embryo development was confirmed by the good correlation between *BMP15* and embryo growth speed, as well as by the multivariable logistic regression analysis. Indeed, *BMP-15* is an oocyte quality-related gene, playing a fundamental role in normal follicular growth, ovulation, and fertilization: increased *BMP-15* levels in FF [53] and in CCs [11] were associated with embryos having good competence for implantation and pregnancy. *BMP-15* is also involved in functional changes of GAP junctions and the downregulation of *Cx43* expression in granulosa cells [24]. Indeed, previous clinical data showed that CCs reducing *Cx43* expression at the time of oocyte collection provided a good embryo competence [54]. In agreement with previous studies, our observations suggest that reduced levels of *Cx43* expression in CCs, coupled with high *BMP-15* mRNA levels, can be considered a marker of embryo competence.

As some proteins are good bi-directional indicators in the communication between the oocyte and the somatic cells surrounding it, we carried out a preliminary analysis aimed at evaluating the metabolic competence of CCs. Interestingly, previous studies on bovine cumulus-oocyte complexes demonstrated that a metabolic consequence of recombinant human *BMP15* addition is the modulation of the oocyte oxidative phosphorylation, acting via CCs [55].

Up to now, no study ever investigated the metabolic consequences of high *BMP15* levels in the human cumulus-oocyte complex, notwithstanding that, as member of the transforming growth factor β (TGF-β) superfamily, also *BMP15* could exert a metabolic effect similar to TGF-β, which is a key modulator of the respiratory chain [56]. Our approach found interesting novel data related to CCs metabolism. In fact, in the CCs corresponding to embryos with arrested development, we detected a significantly decreased expression of both *COXII* and *MT-ATP6*, markers of mitochondrial transcription activity contributing to the modulation of respiratory chain and ATP synthesis. Indeed, *COXII* mRNA levels were significantly higher in the CCs corresponding to embryos that reached the expanded blastocyst stage and even the logistic regression analysis confirmed a significant, direct correlation between embryo progression to blastocyst and *COXII* gene expression. As mitochondrial-encoded proteins are required for the formation of active respiratory complexes, this observation unveils the importance of mitochondria in supporting human embryo growth. A downregulation of *COXII* in CCs, in fact, is associated to reduced ATP levels, decreased mitochondrial membrane potential, and enhanced production of ROS [57]. Likewise, a diminished *MT-ATP6* gene expression may result in reduced ATP synthesis rate [58]. The observed decrease of *COXII* and *MT-ATP6* gene expression in CCs in association with embryo developmental arrest suggests that an impaired mitochondrial function in CCs could negatively affect the development potential of embryos since the earliest stages of growth.

Moreover, it was previously reported that the gene expression of *HO-1* was significantly higher in lower quality, arrested embryos, consistently with a dysfunctional state of mitochondria [59]. We previously reported that the presence of an oxidative stress status inducing a significantly higher *HO-1* expression in CCs may impair oocyte competence [17], suggesting that an excessive ROS production can trigger oocyte damage. The present study further supports the role of ROS, as it shows that a higher expression of *HO-1* in CCs is also associated with a higher likelihood of inadequate embryo development.

Notwithstanding the small sample size, the data of gene expression relative to the embryos that achieve the stage of early or not fully expanded blastocyst show a trend similar to what is observed for the expanded blastocyst on day 5 group, suggesting that also these embryos can implant.

A relevant finding of the current study is the evidence that the expression of genes encoding for some oocyte-secreted factors or reflecting mitochondrial status in CCs are significantly related to the morphokinetic parameters of the corresponding embryo. This finding strongly supports the concept that the follicular microenvironment affects not only oocyte maturation, but also the early embryo growth: in fact, embryo development in the first 3 days is almost exclusively due to the expression of oocyte DNA and to metabolic resources of oocyte cytoplasm [58]. Actually, Hammond et al. reported that the expression of CCs specific genes was significantly associated with the achievement of the blastocyst stage on day 5, but differently from our findings, none of these genes was found to significantly correlate with TLS-acquired parameters [39]. On the other side, Scarica et al. observed that a slower embryo growth was associated with developmental arrest and that the assessment of calcium/calmodulin-dependent protein kinase 1D (*CAMK1D*), prostaglandin-endoperoxide synthase 2 (*PTGS2*) and hyaluronic acid synthase 2 (*HAS2*) gene expression in CCs could boost the predictive power of TLS when the two approaches were combined [60].

Despite the limited number of patients considered in this study, gene expression of both *BMP15* and *COXII* resulted strongly associated with a higher probability of blastocyst development on day 5 at multivariable analysis, independently

from embryo morphokinetic. This suggests that although early embryo development in the first 3 days relies mostly on resources belonging to the oocyte, the follicular environment where the oocyte develops may partially influence embryo competence to become a blastocyst.

In conclusion, our study shows that embryo morphokinetics, in particular in the first 3 days of in vitro growth, as well as the expression of *BMP15* and *COXII* genes in the corresponding CCs, is able to predict whether an embryo will timely reach the expanded blastocyst stage or will prematurely undergo developmental arrest. ROC curves identified the cut-off values of t2 < 26.25 for kinetics and *COXII* > 0.3 for gene expression as optimal thresholds to predict a timely blastocyst development; this observation warrants further testing in order to check its potential for embryo selection and implantation prediction in the routine clinical practice. Our data suggest the possibility of developing integrated prediction models and algorithms for early embryo selection at the cleavage stage including both morphokinetic and molecular parameters, even if time-lapse technology is not routinely used in all IVF laboratories for embryo monitoring as support in embryo selection.

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Authors' contribution A.R., S.C., and L.B. conceived the study; A.C. and N.D.S. enrolled the patients; S.C., C.M., and C.P. were responsible for the time-lapse data acquisition and analysis; L.B. and C.M. performed real-time polymerase chain reaction experiments; L.C. performed the statistical analysis and S.C. contributed to the statistical analysis; S.C. and L.B. contributed to the validation, the interpretation of the results, the writing and editing of the manuscript; F.S., G.G., C.B., and A.R. contributed to the final interpretation of the data and editing of the manuscript. All authors gave their final approval.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Development of a Machine Learning algorithm based on early morphokinetics for human blastocyst development prediction: a retrospective analysis of 575 cleavage-stage embryos.

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Abstract

Study question: Can morphokinetic features included into Machine Learning (ML) algorithms identify cleavage-stage embryos with the best chance to reach the expanded blastocyst stage on day 5?

Summary answer: A ML algorithm based on early morphokinetic features can identify cleaving embryos that will reach the expanded blastocyst stage on day 5.

What is known already: To date, the conventional morphology assessment of cleaving human embryos has a limited predictive power on further embryo developmental potential. The morphokinetic analysis using Time-Lapse systems (TLS) was introduced in order to provide a new tool to identify dynamic biomarkers of embryo quality. More recently, ML approach has been applied for the analysis of specific embryo-related features, aiming at developing predictive algorithms to assess the embryo development potential.

Study design, size, duration: We retrospectively analysed 575 embryos obtained from 80 women aged 25-42 years, with normal BMI, AFC≥8, day 3 FSH<12 IU/l, AMH>2.5 ng/ml, no diagnosis of polycystic ovary syndrome or endometriosis. These patients underwent IVF at our IVF Unit between March 2018 and March 2020; their embryos were cultured using the Geri plus® TLS and a single blastocyst transfer was performed.

Participants/materials, setting, methods: A total number of 29 morphological and morphokinetic parameters were considered to build six different ML algorithms. The performance to assess which was the best-fitting algorithm was calculated using the ROC curve considering accuracy (% of embryos correctly classified by the algorithm), Cohen-kappa coefficient (measurement of the agreement among features), mean number of TP

(embryos correctly classified as undergoing developmental arrest), mean number of TN (embryos uncorrectly classified as progressing to the blastocyst stage).

Main results and the role of chance: Overall, 210 embryos progressed to the expanded blastocyst stage on day 5 (BL group), whereas 365 displayed developmental delay or arrest at any stage (nBL group). Among the six different algorithms, the best-fitting algorithm was obtained using the *Kbest* features selection approach combined with a *Random Forrest* evaluation strategy. This algorithm was based on 7 variables: embryo morphological score on day 2, pronuclear fading time (tPNf), completion time of cleavage to two, four and eight cells (t2, t4, and t8 respectively), time intervals t4-t3 and t8-t4. The algorithm showed an AUC of 0.78, with an accuracy of 0.73, a Cohen-kappa of 0.41, a mean TP number of 302/365 embryos in the nBL group and a mean TN number of 120/210 embryos in the BL group. Mean false positive (FP) and false negative (FN) numbers were of 63 and 90.2, respectively.

Limitations, reasons for caution: The results obtained in this study may not be generalizable to patients with other clinical characteristics, to other time-lapse systems or different laboratory settings. The predictive power of the algorithm should be validated prospectively on a larger number of embryos.

Wider implications of the findings: The current study represents a preliminary analysis for the development of hierarchical predictive models for embryo assessment based on their developmental potential, that embryologists will be able to apply as a support for decision-making.

Keywords: Machine learning - Prediction algorithm - Time-lapse - Embryo selection - IVF

Introduction

The ability to select the embryo with the best implantation potential is a critical factor for the success of IVF treatments. Routinely, embryologists select embryos by a static observation at specific time point of their development either on day 3 or day 5 of in vitro culture (Glujovsky et al., 2016). In fact, the choice of the most suitable embryo to transfer can be improved by extended culture of human embryos to the blastocyst stage (Gardner et al., 1998). In the last decades, elective single blastocyst transfer has progressively become a widespread clinical strategy to reach a fairly good pregnancy rate with very low risk of twin pregnancy (Glujovsky et al., 2016). Notably, the key point is how to select those with the best probability to implant among the available blastocysts. However, an objective method of embryo assessment is still lacking in human embryology, as conventional morphology can provide only a limited predictive power on embryo developmental potential (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011) in non-Preimplantation Genetic Testing (PGT) cycles. In addition, embryo morphological assessment can be highly affected by inter and intra- observer variation and performed by a wider range of different grading systems among the IVF laboratories, thus the consistency and reproducibility are now not guaranteed among the different IVF laboratories (Storr et al., 2017). For this reason, several molecular and biochemical strategies have been proposed also by our group in order to identify novel markers to improve oocyte and embryo selection (Bergandi et al., 2014; Revelli et al 2017; Canosa et al., 2017). More recently, embryo morphokinetic analysis has been introduced, joined to conventional morphological evaluation, with the aim of providing a new tool to identify predictive biomarkers of embryo quality (Meseguer et al., 2011). Time-lapse monitoring of embryo development has been claimed to improve embryo selection (Wong et al., 2010) and IVF outcome by some authors (Pribenzsky et al., 2017), whereas its usage is disputable according

to others (Polanski et al., 2014; Armstrong et al., 2015; Rakowsky et al., 2015; Chen et al., 2017). Even more importantly, its efficacy may partially due to the unperturbed culture conditions rather than to the application of morphokinetic data (Swain et al., 2019). Overall, there is insufficient good-quality evidence of differences in live birth or ongoing pregnancy, miscarriage and stillbirth, or clinical pregnancy to choose between time-lapse system, with or without embryo selection software, and conventional incubation (Armstrong et al., 2019). Consequently, it is still difficult to predict the overall probability of a successful pregnancy for each patient and to fully understand the cause of each failure. In this scenario, in recent years Artificial Intelligence (AI) has experienced rapid growth in various fields, including reproductive medicine, as a new tool to identify biomarkers of gamete and embryo quality, thus representing a pivotal solution to current dilemmas (Wang et al., 2019; Zaninovic et al., 2019, 2020). Using a large quantity of data, predictive or classification algorithms have been developed in order to improve the treatment and prognosis for infertile patients (Chavez-Badiola et al., 2020; Fernandez et al., 2020). Among AI approaches, Machine Learning (ML) represents one of the most common to build algorithms for pattern recognition and feature selection with the aim of accurately predict a new event based on previously acquired experience on the same field (Camacho et al., 2018; Goyal et al., 2020). Based on these data, AI represents an intriguing approach to optimize embryo selection and improve IVF outcome. Aim of the present study was to develop a Machine Learning model for features selection and embryo classification based on early embryo-related variables, including morphological and morphokinetic data obtained using a time-lapse system to identify cleavage stage embryos with the best chance to develop to the expanded blastocyst stage on day 5.

Material and methods

Patients

The study was designed as a preliminary retrospective analysis of 575 embryos obtained from 80 women (mean age: 35.31±3.5, range 25-42) with normal body mass index (BMI 18–25), ovarian reserve markers (serum day 3 FSH < 12 IU/l, antral follicle count, AFC≥8, and anti-mullerian hormone, AMH>2.5 ng/ml) suggesting a normal responsiveness to gonadotropin stimulation (Revelli et al., 2020) and undergoing single blastocyst transfer at our IVF Unit between March 2018 and March 2020. Patients with PCOS, endometriosis and/or with unfavourable markers of ovarian reserve (AMH < 0.5 ng/mL) were excluded from recruitment (Revelli et al., 2016). The patients' clinical characteristics and the outcome of controlled ovarian stimulation (COS) were recorded, including the total dose of exogenous FSH, the peak estradiol (E2) level, the number of retrieved oocytes, the ovarian sensitivity index (OSI = retrieved oocytes × 1000/total gonadotropin dose) (Huber et al., 2013), the fertilization rate, the cleavage rate, the blastocyst formation rate, and the clinical (ultrasound visualization of the gestational sac) pregnancy rate (CPR) per embryo transfer (ET). The study was carried out in accordance with the Declaration of Helsinki and was authorized as a retrospective observational study by the local Ethical Committee (number: 0056908). A signed, written informed consent was obtained from all patients.

Controlled ovarian stimulation and oocyte retrieval

The gonadotropin-releasing hormone (GnRH)-agonist "long" protocol with recombinant FSH (Gonal-F®, Merck, Germany) at individually tailored daily dose (100–300 IU s.c.) was used to carry out controlled ovarian stimulation (COS). Circulating E2 dosage assessment and transvaginal US examination were performed every second day from stimulation day 7 in order to monitor follicular growth. The dose of FSH was adjusted accordingly. A single

s.c. injection of 10,000 IU hCG (Gonasi HP, IBSA, Switzerland) was administered to trigger ovulation, when at least two follicles reached 18 mm mean diameter, with appropriate E2 levels. US-guided oocyte retrieval (OPU) was performed 35–37 h after hCG trigger under local anaesthesia (paracervical block). Follicular fluid was aspirated and immediately observed under a stereomicroscope. Cumulus-oocyte complexes (COCs) were washed in buffered medium (Flushing medium, Cook Ltd., Ireland) and within 4 h from OPU oocytes were inseminated using conventional IVF or microinjected according to the quality of the semen sample. If performing ICSI, 2 h after OPU, oocytes and CCs were separated from each individual COC by gently pipetting in 40-µl HEPES buffered medium containing 80 IU/ml hyaluronidase (Synvitro Hyadase, Origio Medicult, Denmark) (Canosa et al., 2017).

Preparation of semen samples, ICSI, and fertilization check

Sperm concentration, motility, and morphology were assessed according to the World Health Organization guidelines (WHO laboratory manual 5th ed., 2010). Semen samples were prepared using density gradient centrifugation in order to select normally motile morphologically normal spermatozoa. Oocytes were inseminated using conventional IVF or microinjected according to the quality of the semen sample within 4 h from OPU. Normal fertilization was confirmed when the presence of two pronuclei (2PN) and the extrusion of the second polar body were observed 16–18 h after injection.

Time-lapse embryo culture

Embryos were cultured using the Geri plus® TLS (Genea Biomed, Germany) with integrated embryo monitoring system in microwells (one zygote/microwell), as previously described (Canosa et al., 2020). The dish format allowed following each embryo individually even if all embryos shared a common 80-µl drop of medium. Embryos were cultured in pre-equilibrated Cleavage medium (Cook, Ireland) overlaid with mineral oil up to day 3; at this

stage, a change of medium was performed and a new one (Blastocyst medium, Cook, Ireland) was added and kept until the blastocyst stage. Bright field images captured by the Geri plus® system were acquired every 5 min from the time of fertilization until the time of embryo transfer (ET), cryopreservation, or discharge. Embryo morphological evaluation was firstly performed on day 2 using the Integrated Morphology Cleavage Score (IMCS) (Holte et al., 2007); embryo selection for ET was performed on day 5 considering both conventional morphology (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011) and morphokinetic parameters (Meseguer et al., 2011). All the videos collected by TLS were analysed, and the following morphokinetic parameters (times) were considered, according to the ESHRE Working group on Time-lapse technology (2020): pronuclear appearance (tPNa), pronuclear fading (tPNf), completion of cleavage to two, three, four, and eight cells (t2, t3, t4, and t8 respectively), time intervals tPNf-tPNa, t2-tPNf, t3-t2, t4-t3, t4-t2, and t8-t4. Embryos that progressed to the expanded blastocyst stage on day 5 (score 3 according to Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011) were included in the blastocyst group (BL group; n = 210), whereas those undergoing developmental arrest or that progressed to the morula stage, the early or not fully expanded blastocyst stage on day 5 (score 1 or 2) were included in the not-expanded blastocyst group (nBL group; n = 365), as previously described (Canosa et al., 2020b). Blastocyst assessment was performed at the same time interval (116 ± 2 h after oocyte insemination) for all embryos. Eighty expanded blastocysts were transferred in utero (one per patient): 62 as fresh embryo transfer and the remaining 18 as frozen embryo transfer. ET was performed using the soft catheter Sydney Guardia (Cook, Australia) under transvaginal US guidance, applying the method previously published by our group (Revelli et al., 2016).

Dataset preparation, feature selection and classification strategy

To perform an adequate embryo classification, a high-quality training dataset was obtained. For the first step of feature selection, any redundant information was deleted to a total number of 29 features (age, BMI, day 3 FSH, day 3 AMH, AFC, years of infertility, total dose of FSH, peak estradiol, OSI, number of retrieved oocytes, number of mature oocytes, maturation rate, number of fertilized oocytes, fertilization rate, number of cleaved embryos, cleavage rate, number of blastocysts, blastocyst rate, embryo IMCS, tPNf, t2, t3, t4, t8, t2-tPNf, t3-t2, t4-t3, t4-t2, t8-t4). This number was established in order to reduce the overfitting (less possibility of making decisions based on redundant data) and training time (the algorithm trains faster), and improve the accuracy (lower misleading data). Two different approaches have been performed: the *sfm*, meta-transformer for selecting features based on importance weights, and the *kbest* to select features according to the k highest scores. For the second step of embryo classification, three different strategies have been used: *Random Forrest*, *Logistic regression* and *Boosting*. A total number of six algorithms were obtained combining the previously described approaches after training on 90 % samples, tested on 10 % of samples and a 10-fold cross-validation. For each run 30 replicates were implemented so that 300 runs for features selection and for classification have been performed.

Heat maps and ROC curves

The correlations between all variables considered have been depicted as a heat map using a colour scale. The resulting graphs were represented in a matrix format where coefficients of correlations were reported and ranged from - 0.6 to 1. The performance of the best-fitting algorithm was calculated using the receiver operating characteristic (ROC) curve generated by plotting the area under the curve (AUC) to the progressive increase of the number of features considered. AUC may range from 0.5 to 1.0, which represents the predictive power

of the algorithm. An AUC of 0.5 describe completely random choices whereas an AUC of 1 represents perfect discrimination. As a result, AUC was the most appropriate approach to rank embryos according to their likelihood of develop into an expanded blastocyst. For each of the training-testing runs, the AUC has been calculated on the testing data. The final AUC has been reported as the mean AUC ± SEM over ten separate training-testing runs. The best-fitting algorithm for prediction of blastocyst development was described by the following characteristics: Accuracy = % of embryos correctly classified by the algorithm; Cohen-kappa = measurement of the agreement between the features; TP (true positive) = mean number of embryos correctly classified in the nBL group; FP (false positive) = mean number of embryos wrongly classified in the nBL group; TN (true negative) = mean number of embryos correctly classified in the BL group; FN (false negative) = mean number of embryos wrongly classified in the BL group.

Results

Patients

Table 1 summarizes the clinical characteristics of the 80 patients and the outcome of their IVF cycles. As expected, the age and ovarian reserve markers were of good prognosis patients; indeed, the retrieved oocytes mean number was 12.1 ± 5.3, the mean number of embryos and blastocyst obtained was 7.2 ± 2.9 and 4.6 ± 2.8, respectively. The clinical PR/ET was 40%, in line with the predicted outcome after transfer in utero of a single expanded blastocyst in such patients. Overall, 575 embryos were obtained in vitro: 210 progressed to the expanded blastocyst stage on day 5 and were included in the blastocyst group (BL group), whereas 365 underwent developmental arrest or progressed to the morula stage, the early or not fully expanded blastocyst stage on day 5 and were included in the not-expanded blastocyst group (nBL group).

Correlation analysis between the considered features

The correlations between all the features considered are represented as heat maps in Figure 1A. The analysis identified two main clusters of positive correlations (red dots), one on the upper left of the panel related to clinical characteristics and one on the lower right related to morphokinetic parameters. As expected, clinical characteristics such as peak E2, OSI, number of retrieved oocytes and number of mature oocytes were strongly correlated as well as all times of cell divisions up to the eight-cell stage. In addition, the number of the blastocysts obtained in vitro showed correlation to the total number of oocyte yield and the number of mature oocytes. Interestingly enough, the embryo IMCS score evaluated on day 2 was inversely correlated to the embryo morphokinetics at the cleavage stage.

Machine learning algorithms for blastocyst identification

A total number of six algorithms were obtained and represented as ROC curves (Figure 1B). The best-fitting algorithm was obtained using the *kbest* feature selection approach combined with a *Random Forrest* classification strategy. The algorithm included a total number of 7 embryological features (embryo IMCS, tPNf, t2, t4, t8, t4-t3, t8-t4). The algorithm was described by an AUC of 0.78 with an accuracy of 0.73, a Cohen-Kappa of 0.41, a mean true positive (TP) number of 302 out of an overall number of 365 embryos classified in the nBL group and a mean true negative (TN) number of 120 out of an overall number of 210 embryos classified in the BL group. Mean false positive (FP) and false negative (FN) number were of 63 and 90.2, respectively.

Discussion

The introduction of artificial intelligence (AI) in reproductive medicine, have opened a revolutionary scenario for the generation of complex algorithms handling an extremely large amount of clinical data (Wang et al., 2019). Aim of the present study was to develop a ML algorithm for features selection and embryo classification based on clinical and early embryological data, including morphokinetics obtained using a time-lapse system to identify cleavage stage embryos with the best chance to develop to the expanded blastocyst stage on day 5. Our primary endpoint derives from the evidence that the transfer of fresh, slow-growing blastocysts may result in lower implantation and clinical pregnancy rate compared to the transfer of fully expanded day 5 blastocysts (Shapiro et al., 2001; Barrenetxea et al., 2005), probably due both to a reduced embryo competence and to a loss of embryonic-endometrial synchrony (Franasiak et al., 2018). Moreover, the transfer of a fresh early blastocyst on day 5 was found to obtain success rates comparable to the transfer of a thawed expanded blastocyst cryopreserved on day 6 from the same cohort (Tannus et al., 2019), suggesting that embryo kinetic is more important than the embryo developmental stage itself in determining IVF outcome. In fact, in cases where only early or not fully expanded blastocysts are available on day 5, extending culture until cryopreservation on day 6, and subsequent transfer after thawing may result in successful pregnancy. Taken together, these observations lead us to consider for our analysis the fully expanded blastocyst stage as the ideal reference point that most likely relates to higher embryo implantation. Herein, the retrospective assessment of 575 embryos generated in vitro from patients undergoing single blastocyst transfer revealed that all morphokinetic features at the cleavage stage up to eight-cells showed significant correlation. This in line with our previous observation showing that embryos able to achieve the expanded blastocyst stage on day 5 can be predicted quite early looking at the duration of the first cytokinesis, being characterized by a specific trend in

cellular kinetic already during the first 3 days of development (Canosa et al., 2020). Interestingly, morphological embryo assessment on day 2 using the evidence-based score named IMCS (Holte et al., 2007) resulted inversely correlated to the embryo morphokinetic up to the eight-cell stage, suggesting that our morphological evaluation showed consistency with embryo potential to progress through the cleavage stage. To date, however, a general consensus on the correlation between conventional morphology and early embryo morphokinetic is still lacking, as the association between a higher expansion grades, the quality of the inner cell mass (ICM) and the trophectoderm (TE) with shorter time to start of blastulation, expansion and hatching was demonstrated only in euploid blastocysts (Minasi et al., 2016). Further, using ML methods, we developed an algorithm to apply for features selection and embryo classification, according to the ability to progress to the expanded blastocyst stage on day 5. Differently form a multivariable analysis by classic statistics, ML can build complex algorithms obtained from the analysis of specific data sets, then applied to future similar situations. This approach may take into account links and associations among the considered variables, thus providing unbiased robust models for future predictions (Barnett-Itzhak et al. 2020). The best-fitting algorithm was developed from a total number of seven embryological variables, as follows: embryo IMCS, tPNf, t2, t4, t8, t4-t3, t8-t4. Interestingly enough, the difference between IMCS and other scoring methods is that IMCS was constructed comparing the morphology of surely implanted embryos (dizygotic twin pregnancies after DET) vs. surely non-implanted embryos (no pregnancy after DET), and was therefore based on the evidence of implantation and clinical pregnancy. Indeed, IMCS was incorporated into a complex prediction model for IVF outcome shown to predict live birth with a remarkably good precision (Vaegter et al., 2017). Finally, the majority of embryo morphokinetic parameters evaluated at the cleavage stage were selected by the algorithm, suggesting that early embryo cytokinesis may be used as precocious

markers of embryo competence to progress to the expanded blastocyst stage, as previously described (Wong et al., 2010; Canosa et al., 2020). The presence in the algorithm of many embryological features suggested that embryo developmental kinetic had the strongest predictive power, independently from patient' and cycle characteristics they derive from. However, previous studies reported that culture conditions, patient characteristics and treatment might influence the timings of embryo development. Kirkegaard et al. (2016) observed a moderate variation in terms of developmental dynamics among different embryos of the same patient, suggesting a patient-specific clustering in embryo morphokinetics. In addition, no single patient- and treatment-related factor was found to systematically influence the timings from cleavage to blastocyst stage, which indicates that no individual patient-related factor can be identified that separately explains the clustering throughout the entire developmental stages. Furthermore, the blastocyst parameters were more affected by patient-related factors than cleavage stage parameters. Consequently, embryos from the same cohort should be considered as dependent observations, affected by patient-specific confounding factors. Surprisingly, in the present analysis, female age was not selected as a reliable feature, despite it has been observed that maternal age may affect early cytokinesis at the cleavage stage, with significantly shorter times in younger patients (Akhter et al., 2017; Akarsu et al., 2017; Faramazi et al., 2019). In addition, it is well known that maternal age is strongly correlated with the proportion of aneuploid oocytes, which, at baseline, are estimated around 20% in young women, but this increases exponentially from 30 to 35 years, reaching on average 80% by 42 years (Capalbo et al., 2017). Thus, pre-implantation genetic testing for aneuploidy (PGT-A) represent a tool to identify euploid embryos during IVF, in particular in infertile women of advanced maternal age, with a history of recurrent miscarriages and/or IVF failures (Vaiarelli et al., 2016). Embryo development dynamics was demonstrated to be affected by chromosome aneuploidy and further analysis of the

chromosome content revealed higher differences when the complexity in the chromosome disorders is increased (Nogales et al., 2017). Thus, the absence of maternal age in a complex predictive algorithm from embryo classification should be further investigated with an adequately powered sample size, focusing on implantation potential of the embryo, which was however beyond the aim of the present study. Overall, the performance of the algorithm was calculated with an area under the curve (AUC) of 0.78 and an Accuracy of 0.73, suggesting the ability to correctly identify embryos progressing to blastocysts in the 73% of cases, according to the previously selected features. The combination of embryo score based on the classical morphological parameters and early morphokinetic times was also used by Carrasco et al. (2017) for the development of a decision tree in which the morphological score resulted the most relevant feature, leaving morphokinetic parameters as secondary. The most relevant morphokinetic parameters to predict implantation showed significant differences for t2, t3, t4, t7, t8, s2 and cc2 of which t2, t4, t8, t4-t3 (=s2) are in common with the present study. However, the morphokinetic parameters of better prognosis should be identified according to the working conditions of the specific laboratory, as previously mentioned. The main limitations of this study derive from the lack of a prospective validation of the algorithm. In addition, these results may not be generalized to other patient populations, time-lapse systems or laboratory settings. However, the current study represents a preliminary analysis for the development of hierarchical models for embryo classification based on the probability of implantation using known implantation data (KID). In conclusion, in our hands, the morphokinetic parameters make it possible to differentiate the embryos with the best chance to progress to the expanded blastocyst stage together with the classical morphological score. In the future, ML approach for the optimization of embryo selection may improve pregnancy chance in IVF by helping in clinical decision making as well as for quality control.

TABLES

	Patients (n=80)
Age (years)	35.3 ± 3.5
BMI (kg/m^2)	24.2 ± 4.8
Basal (day 3) FSH (IU/l)	7.3 ± 3.9
AMH (ng/ml)	5.4 ± 4.2
AFC (n)	17.5 ± 8.3
Infertility (years)	2.9 ± 1.5
Total exogenous FSH (IU)	2137.1 ± 900
Peak E2 (pg/ml)	2246.4 ± 1267.4
Endometrial thickness (mm)	10.6 ± 2.3
OSI (n)	6.8 ± 4.7
Retrieved oocytes (n)	12.1 ± 5.3
Maturation rate (%)	85.9 ± 14.9
Fertilization rate (%)	73.7 ± 17.7
Cleaved embryos (n)	7.2 ± 2.9
Cleavage rate (%)	98.6 ± 4.9
Blastocyst obtained (n)	4.6 ± 2.8
Blastocyst formation rate (%)	63.8 ± 24.7
Clinical Pregnancy rate/ET %(n)	40 (32/80)

Table 1. Patients' clinical characteristics and IVF outcome. Data are shown as mean ± standard deviation, with the only exception of the last variables which are expressed as a percentage. BMI body mass index, AFC antral follicle count, FSH follicle-stimulating hormone, E2 estradiol, OSI ovarian sensitivity index = number of retrieved oocytes × 1000/total FSH dose.

FIGURES

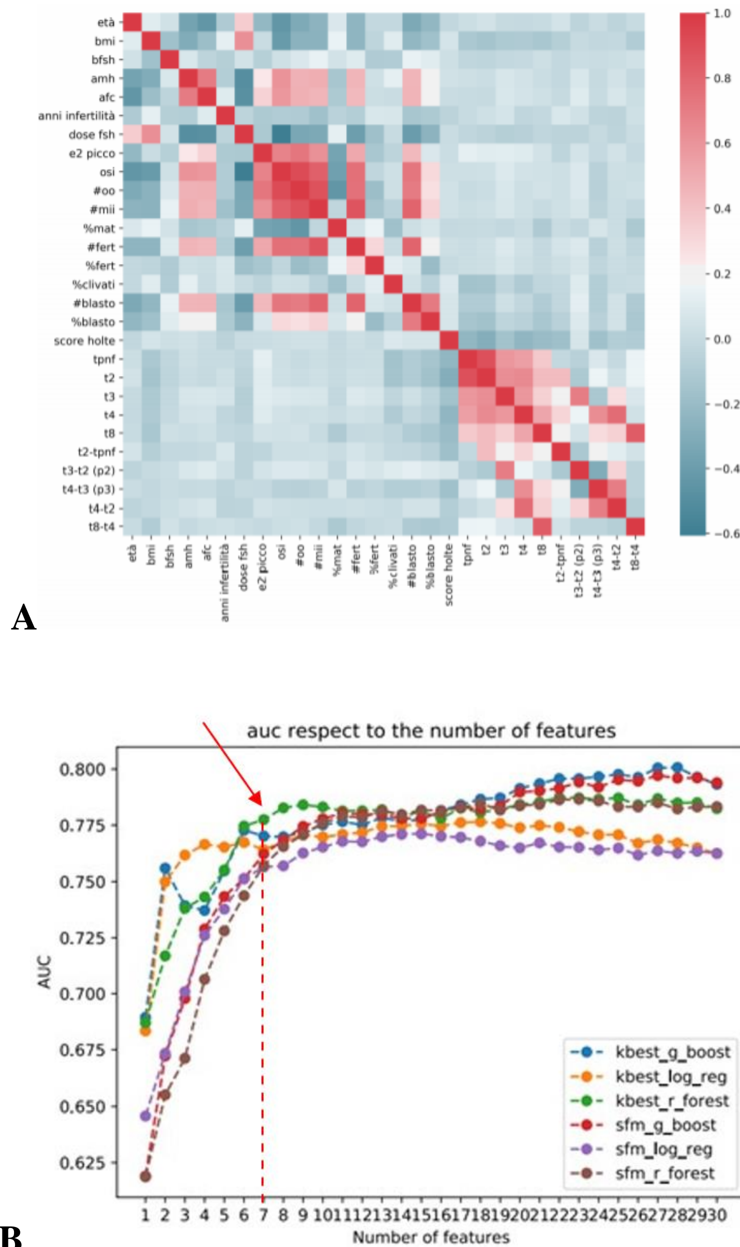

Figure 1. Heat map of correlation analysis and ROC curves for prediction of blastocyst development. Each row and column represent a clinical, morphological or morphokinetic variable considered in the dataset of 29 features. According to the coefficient, correlations have been marked as positive or negative using a scale of red-blue colours (A). ROC curves were obtained by the combinations of feature selection and classification strategies. AUC = area under the curve was calculated versus the increasing number of features considered at each run (B).

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3. Conclusions

Aim of the present thesis was to evaluate whether Time-Lapse technology (TLT) and innovative computer-based approaches of Artificial Intelligence (AI) may represent novel strategies to optimize early embryo selection and improve pregnancy chance in modern IVF. At first, in our paper entitled *“Impact of the addition of Early Embryo Viability Assessment to morphological evaluation on the accuracy of embryo selection on day 3 or day 5: a retrospective analysis”* (Revelli et al., 2019), we observed that morphokinetic analysis of the developing embryo coupled to conventional morphology may be useful in improving the embryo selection when embryo transfer (ET) is scheduled on day 3, obtaining clinical results comparable to blastocyst transfer on day 5. However, in the last decades the transfer of a single blastocyst is becoming the gold standard in IVF to both maximize the pregnancy chance and reduce the twinning risk. Thus, in our paper entitled *“Morphokinetic analysis of cleavage stage embryos and assessment of specific gene expression in cumulus cells independently predict human embryo development to expanded blastocyst: a preliminary study”* (Canosa et al., 2020), we observed that embryo morphokinetics, in particular in the first 3 days of in vitro growth, as well as the expression of oocyte quality genes such as BMP15 and COXII in the corresponding cumulus cells (CCs), is able to predict whether an embryo will timely reach the expanded blastocyst stage or will prematurely undergo developmental arrest. Our data opened the possibility of developing integrated prediction models and algorithms for early embryo selection at the cleavage stage including both morphokinetic and other embryological parameters. The extremely rapid increase in the quantity of data obtained in the last years faced the need to develop complex predictive or classification algorithms to improve clinical outcomes in modern medicine. Among computer-based approaches, Artificial Intelligence (AI) and Machine Learning (ML), in

particular, represent intriguing strategies to build algorithms for feature selection and class classification with the aim of accurately predict a new event based on previously acquired experience. In our last research *"Development of a Machine Learning algorithm based on early morphokinetic for human blastocyst development prediction: a retrospective analysis of 575 cleavage-stage embryos" (Canosa et al., unpublished),* we developed a ML algorithm containing seven morphological and morphokinetic variables able to classify embryo progressing to the expanded blastocyst stage with an accuracy of 73%, suggesting the possibility to further improve this strategy with the development of hierarchical models for a more accurate embryo classification. In the future, the introduction of ML approaches in the IVF labs for the optimization of embryo selection may represents a revolutionary scenario able to dramatically change the decision-making process and the personalization of the IVF treatments.

4. Other publications during the PhD

4.1 Research articles

1. **Canosa S**, Paschero C, Carosso A, Leoncini S, Mercaldo N, Gennarelli G, Benedetto C, Revelli A. Effect of a combination of Myo-Inositol, Alpha-Lipoic acid, and Folic acid on oocyte morphology and embryo morphokinetics in non-PCOS overweight/obese patients undergoing IVF: a pilot, prospective, randomized study. J Clin Med 2020.
2. Carosso A, Revelli A, Gennarelli G, **Canosa S**, Cosma S, Borella F, Tancredi A, Paschero C, Boatti L, Zanotto E, Sidoti F, Bottino P, Costa C, Cavallo R, Benedetto C. Controlled ovarian stimulation and progesterone supplementation affect vaginal and endometrial microbiota in IVF cycles: a pilot study. J Assist Reprod Genet 2020.
3. **Canosa S**, Bergandi L, Carosso AR, Paschero C, Gennarelli G, Silvagno F, Benedetto C, Revelli A. Human Recombinant FSH and Its Biosimilars: Clinical Efficacy, Safety, and Cost-Effectiveness in Controlled Ovarian Stimulation for In Vitro Fertilization. Pharmaceuticals (Basel). 2020.
4. Masciovecchio M, Scarafia C, Razzano A, **Canosa S**, Gennarelli G, Carosso AR, Benedetto C, Revelli A. Is guided, targeted information about the risks of twin pregnancy able to increase the acceptance of single embryo transfer among IVF couples? A prospective study. J Assist Reprod Genet 2020.
5. Silvestris E, De Palma G, **Canosa S**, Palini S, Dellino M, Revelli A, Paradiso AV. Human Ovarian Cortex biobanking: A Fascinating Resource for Fertility Preservation in Cancer. Int J Mol Sci 2020.

6. Revelli A, Gennarelli G, Sestero M, **Canosa S**, Carosso A, Salvagno F, Pittatore G, Filippini C, Benedetto C. A prospective randomized trial comparing corifollitropin-α late-start (day 4) versus standard administration (day 2) in expected poor, normal, and high responders undergoing controlled ovarian stimulation for IVF. J Assist Reprod Genet 2020.
7. Gennarelli G, Carosso A, **Canosa S**, Filippini C, Cesarano S, Scarafia C, Brunod N, Revelli A, Benedetto C. ICSI Versus Conventional IVF in Women Aged 40 Years or More and Unexplained Infertility: A Retrospective Evaluation of 685 Cycles with Propensity Score Model. J Clin Med 2019.
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9. Revelli A, **Canosa S**, Bergandi L, Skorokhod OA, Biasoni V, Carosso A, Bertagna A, Maule M, Alfieri E, D'Eufemia MD, Evangelista F, Colacurci N, Benedetto C. Oocyte polarized light microscopy, assay of specific follicular fluid metabolites, and gene expression in cumulus cells as different approaches to predict fertilization efficiency after ICSI. Reprod Biol Endocrinol, 2017.
10. Revelli A, Carosso A, Grassi G, Gennarelli G, **Canosa S**, Benedetto C. Empty follicle syndrome revisited: definition, incidence, aetiology, early diagnosis and treatment. Reprod Biomed Online, 2017.
11. **Canosa S**, Adriaenssens T, Coucke W, Dalmasso P, Revelli A, Benedetto C, Smitz J. Zona pellucida mRNA expression in human oocytes is related to oocyte maturity, zona inner layer retardance and fertilization competence. Mol Hum Reprod, 2017.
12. **Canosa S**, Moggio A, Brossa A, Pittatore G, Marchino GL, Leoncini S, Benedetto C, Revelli A, Bussolati B. Angiogenic properties of endometrial mesenchymal

stromal cells in endothelial co-culture: an in vitro model of endometriosis. Mol Hum Reprod, 2017.

4.2 Abstract at congresses

1. **Canosa S**, Mercaldo N, Carosso AR, Evangelista F, Racca C, Ruffa A, Gennarelli G, Revelli A, Benedetto C. Ovarian Stimulation with rFSH+rLH vs. rFSH alone in poor/suboptimal/normal responders undergoing IVF: real life analysis of 1382 patients stratified for the number of retrieved oocytes. 36th Virtual Eshre Annual Meeting (2020), *accepted as poster presentation.*
2. Gennarelli G, Paschero C, **Canosa S**, Benedetto C[1] and Revelli A[1]. Effect of Myo-Inositol and Alpha-Lipoic Acid on oocyte morphology and embryo morphokinetics: a prospective preliminary analysis of 40 overweight patients undergoing ICSI treatment. 36th Virtual Eshre Annual Meeting (2020), *accepted as poster presentation.*
3. Carosso A, Revelli A, Gennarelli G, **Canosa S**, Cesarano S, Brunod N, Scarafia C, Leoncini S, Boatti L, Zanotto E, Sidoti F, Cavallo R, Benedetto C. Impact of controlled ovarian stimulation on vaginal and endometrial microbiota in IVF cycles: a pilot study. 36th Virtual Eshre Annual Meeting (2020), *accepted as oral presentation.*
4. Parmegiani L, Garello C, **Canosa S**, Granella G, Evangelista F, Monelli G, Guidetti D, Bongioanni F, Filicori M. Universal warming protocol" for vitrified blastocysts: the "coming-out" of "off-label" use of warming kit brands. 36th Virtual Eshre Annual Meeting (2020), *accepted as poster presentation.*

5. Revelli A, Sestero M, **<u>Canosa S</u>**, Filippini C, Pittatore G, Salvagno F, Gennarelli G, Benedetto C. Prospective randomized trial comparing corifollitropin-alfa late start (day 4) vs. corifollitropin-alfa standard start (day 2) in expected poor, normal and high-responders undergoing IVF/ICSI, 35th Eshre Annual Meeting (2019)*, accepted as poster presentation.*
6. Revelli A, **<u>Canosa S</u>**, Bergandi L, Skorokhod OA, Biasoni V, Carosso A, Bertagna A, Maule M, Alfieri E, D'Eufemia MD, Evangelista F, Colacurci N, Benedetto C. Biochemical and genetic markers of oocyte competence to fertilization. 18th World Congress if the Academy of Human Reproduction (2019), *accepted as oral presentation.*
7. **<u>Canosa S</u>**, Marini E, Castiglia S, Rustichelli D, Pinnetta G, Carosso A, Adamini A, Vidoni C, Ferrero I, Isidoro C, Gennarelli G, Bussolati B, Revelli A, Benedetto C, Fagioli F, Mareschi K. Un nuovo metodo di isolamento ed espansione di Cellule Staminali Endometriali per la terapia dell'infertilità. Congresso Nazionale SIFES e MR (2019), *<u>awarded as best oral presentation.</u>*
8. Marini E, **<u>Canosa S</u>**, Castiglia S, Rustichelli D, Pinnetta G, Carosso A, Adamini A, Vidoni C, Ferrero I, Isidoro C, Gennarelli G, Bussolati B, Revelli A, Benedetto C, Fagioli F, Mareschi K. A new method of endometrial mesenchymal stem cell isolation and expansion for the infertility treatment. GISM Annual Meeting (2019), *accepted as poster presentation.*

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